the polypeptide that binds tet operator sequences is a "Tet repressor or mutated Tet repressor." Support for this amendment can be found in canceled claims 3, 6, 17 and 20. Claims 10 and 24 also have been amended to correct a typographical error. New claims 27 and 28 have been added; the text of these claims mirrors claims that have issued in parent application Serial No. 08/486,814, now U.S. Patent No. 5,866,755. The specification has been amended to update the status of the related applications.

For the Examiner's convenience, a copy of the claims as pending after the amendments herein is provided as Appendix A.

No new matter has been added. Applicants request that the amendments to the specification and claims be entered. Amendment of the claims should in no way be construed as an acquiescence to any of the Examiner's rejections and was done solely to more particularly point out and distinctly claim Applicants' invention in order to expedite the prosecution of the application. Applicants reserve the right to pursue the claims as originally filed in this or a separate application(s).

Rejection of Claims 1-11 and 13-26 Under 35 U.S.C. §112, first paragraph

Claims 1-11 and 13,-26 have been rejected under 35 U.S.C. §112, first paragraph, for lack of enablement. The Examiner contends that the specification "does not reasonably provide enablement for *any and all transgenic non-human animals*, whose genome comprises: a first transgene comprising a DNA sequence encoding any and all fusion proteins that inhibit transcription in eukaryotic cells wherein the fusion protein comprises *any polypeptide* that binds to tet operator and that is operatively linked to *any and all transcriptional silencer domains* that inhibit transcription in eukaryotic cells" (emphasis added) Each of the issues raised by the Examiner will be addressed separately below.

I. With regard to "any polypeptide that binds to a tet operator", Applicants point out that the independent claims have been amended to recite that the polypeptide is either a Tet repressor or a mutated Tet repressor. The specification teaches (at least at pages 7-10 and page 30, line 27 through page 31, line 4), that the term "tet repressor" includes "repressors of different class types, e.g., class A, B, C, D or E tet repressors". The sequences and characteristics of these different Tet repressor molecules (including their abilities to interact with different classes of Tet operator sequences) are set forth, for example, in the references disclosed at page 9, each of which has been incorporated by reference into the specification. Furthermore, the specification teaches mutated Tet repressors that have the reverse binding phenotype from the wild-type Tet repressor (*i.e.*, they bind to tet operator sequences in the presence, rather than the absence, of tetracycline). Still further, the specification teaches that different Tet operators may be utilized as targets for the claimed fusion proteins, and the sequences and differing Tet repressor specificities of these Tet operators are set forth in Figure 5. Therefore, the specification fully enables the use of Tet repressors or mutated Tet repressors that bind tet operator sequences in the fusion proteins of the invention.

II. With regard to "any and all transcriptional silencer domains", the Examiner contends that only the use of a transcription silencer domain of a protein selected from the group consisting of v-erbA, the Drosophila Krueppel protein, the retinoic acid receptor alpha, the thyroid hormone receptor alpha, the yeast Ssn6/Tup1 protein complex, the Drosophila protein even-skipped, SIR1, NeP1, the Drosophila dorsal protein, TSF3, SFI, the Drosophila hunchback protein, the Drosophila knirps protein, WT1, Oct-2.1, the Drosophila engrailed protein, E4BP4 and ZF5 is enabled by the specification. However, the Examiner has provided no reasons why the use of other transcriptional silencer domains is not enabled, beyond the general arguments relating to the "unpredictability" of making transgenic organisms (which are addressed in the following section), nor has

the Examiner provided any evidence in support of the position that only these transcriptional silencer domains are enabled. The ability of transcriptional silencer domains to function cross-species, as well as the modular nature of such silencer domains (allowing them to be incorporated into fusion proteins and still maintain their inhibitory function), was established and accepted in the art at the time of the invention. The Examiner has provided no evidence to suggest that transcriptional silencer domains other than those in the aforementioned list would not work in the fusion proteins of the invention. The burden is on the PTO to provide evidence as to why a claimed invention is not enabled. It is Applicants' position that the specification fully enables the use of transcriptional silencer domains, as claimed, in the fusion proteins of the invention.

III. With regard to "any and all transgenic, non-human animals", the Examiner states that "there are several significant limitations to the application of the same methodology of making transgenic animals to different species" and first cites Mullins et al. to support this position, who report 5-70 fold lower yields of recombinant protein in transgenic mice when a construct designed for expression in sheep was used. Mullins et al., however, is an example of successful expression of a transgene in sheep, demonstrating that, at the time of the invention, technology for successfully making transgenic sheep was available in the art. The fact that Mullins et al. observed lower expression in transgenic mice when the identical sheep construct was used in the mice simply indicates that a transgene designed for one species may not be optimal in another species. However, the specification teaches that the design of expression vectors will depend on the particular host cell in which the protein is to be expressed (see page 13, lines 29-31) and various promoters for expression in different species of host cells were known in the art. Moreover, the specification discloses constructs that allow for high level transgene expression in mice, which is what appears to be deficient in Mullins et al.

Further, it should be emphasized that Mullins et al. demonstrate that the transgene was in fact expressed in both animals, just to different degrees.

The Examiner next cites Hammer et al. in support of the "unpredictability" in making transgenic animals. The Examiner relies on Hammer et al. for teaching "that the integration of a transgene into alternative species may result in widely different phenotypic responses even in animals of the same family." However, this reference specifically teaches not only that the expression and activity of the B27 transgene in mice was "physiologically normal", but further suggests that the arthritis phenotype that the researchers were expecting in the mice upon expression of the transgene may not be related to B27 expression at all, and further, that several arthritic diseases readily inducible in rats cannot be induced in mice, regardless of transgene expression (Page 1099, column 2, paragraph 2). This reference neither teaches nor suggests that the phenotypic difference between rats and mice was due to differential expression or activity of the B27 transgene in these animals. In fact, this reference is another example of successful expression of a transgene in two different species, rats and mice.

The third reference cited by the Examiner in support of the "unpredictability" in making transgenic animals is Seidel, which the Examiner relies upon for teaching that:

> "In the case of livestock species....characterizing a transgenic line often is a greater logistical undertaking than making the transgenic founder. Ideally, animals should be evaluated for the transgenic trait as well as for the absence of undesirable side effects in both sexes in both the hemizygous and homozygous transgenic states. Producing homozygous transgenic animals requires mating relatives, resulting in inbreeding. Characterization of transgenic lines takes many years in species with long generation intervals."

These teachings of Seidel at most point out that creating transgenic animals in species other than mice may be more time-consuming or problematic from a logistic standpoint, but the fact that more time may be necessary to make a non-murine transgenic animal or that it may be logistically more difficult does not mean that such animals are not enabled by the invention. In fact, Seidel teach that in five separate studies utilizing cattle, goats,

sheep and swine, between 3.6 and 9.8% of the offspring attained contained the transgene, and between 37.5 and 55.6% of these transgenic offspring expressed the transgene (Page 28, Table 2). Thus, again, the reference cited by the Examiner actually demonstrates that technologies for successfully making transgenic animals in non-mouse species were available in the art at the time of the invention. It would therefore not have required undue experimentation on the part of one skilled in the art to obtain a transgenic animal as claimed in the invention.

Finally, the Examiner cites Ackland-Berglund and Leib as teaching "that the efficacy of tetracycline-controlled gene expression is influenced by cell type." Acklund-Berglund and Leib teach that the expression of a gene of interest operatively linked to the minimal human cytomegalovirus (HCMV) immediate early (IE) promoter and also to seven copies of the Tet operator derived from Tn10 by the tetracycline-controlled transactivator, TetR-VP16, is cell-type dependent. The Examiner appears to be of the opinion that this reported variability is due to cell-type-specific differences in the expression of the transactivator fusion protein. On the contrary, this reference specifically teaches that the differences in expression level are likely due to the activity of the promoter. For example, at page 196, column 2, paragraph 1, the authors state:

> "Although this original system has widespread utility, here we show that it is not functional in all cell types because the activity of the tetO/HCMV IE promoter varies significantly in certain cell lines" (emphasis added).

Therefore, this reference neither teaches nor suggests that the Tet repressor-transcriptional regulator fusion protein has differing activities in different cell types, or that the Tet operator has different binding characteristics in different cell types, but rather teaches that the promoter selected for fusion to the gene of interest, in conjunction with the Tet operator, may result in different levels of expression of the gene of interest in a cell-type-dependent fashion. The specification fully enables one skilled in the art to

adapt the genetic constructs of the invention such that they are tailored to cells of the particular organism (e.g., animal) of choice. The instant specification teaches that the promoter used in operative linkage to the Tet operator(s) and the gene of interest may be selected for optimal expression of the gene of interest in different cell types (see e.g., page 13, lines 29-31) and also provides exemplary regulatory elements to direct expression of the fusion protein in different cell types (see e.g., page 14, lines 6-19).

Furthermore, the teachings in the instant specification with regard to the construction and use of the Tet repressor-transcriptional inhibitor fusion proteins have been successfully applied in a variety of eukaryotic cell types using a closely related system, the tTA system, in which an activator domain rather than an inhibitor domain is used in the fusion protein. Applicants refer the Examiner to:

1) Agarwal *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92:8493-8497, which uses the tTA system in human fibroblasts (see ref. AI in IDS, attachment B);

2) Bergman *et al.* (1995) *Mol. Cell Biol.* 15:711-722, which uses the tTA system is HeLa cells (see ref. AJ in IDS, attachment B);

3) Buckbinder *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:10640-10644, which uses the tTA system in human osteosarcoma cells (see ref. CP in IDS, attachment A);

4) Cayrol and Flemington (1995) *J. Virol.* 69:4206-4212, which uses the tTA system in epithelial cells (see ref. AK in IDS, attachment B);

5) Chen *et al.* (1995) *Cancer Research* 55:4536-4539, which uses the tTA system in colon carcinoma and prostate carcinoma cells (see ref. AL in IDS, attachment B);

6) Gjetting *et al.* (1995) *Biol. Chem. Hoppe-Seyler* 376:441-446, which uses the tTA system in mammary carcinoma cells (see ref. AO in IDS, attachment B);

7) Haase *et al.* (1994) *Mol. Cell. Biol.* 14:2516-2524, which uses the tTA system in human embryonic kidney fibroblasts (see ref. AP in IDS, attachment B);

8) Howe *et al.* (1995) *J. Biol. Chem.* 270:14168-14174, which uses the tTA system in rat pituitary-derived cells (see ref. AR in IDS, attachment B);

9) Miller and Rizzino (1995) *Exp. Cell Res.* 218:144-150, which uses the tTA system in embryonal carcinoma cells (see ref. AS in IDS, attachment B);

10) Resnitzky *et al.* (1994) *Mol. Cell. Biol.* 14:1669-1679, which uses the tTA system in Rat-1 fibroblasts (see ref. CK in IDS, attachment A);

11) Sopher *et al.* (1994) *Mol. Brain Res.* 26:207-217, which uses the tTA system in human neuroblastoma cells (see ref. AU in IDS, attachment B); and

12) Wu *et al.* (1995) *Genes Dev.* 9:2350-2363, which uses the tTA system in NIH-3T3 cells (see ref. AV in IDS, attachment B).

The fact that Tet repressor-transcriptional activator fusion proteins have been demonstrated to regulate target gene expression in a tetracycline-controlled fashion in a variety of cell types when constructed and used in accordance with the teachings of the instant specification indicates that the specification fully enables the construction and use of the Tet repressor-inhibitor fusion proteins of the invention in a variety of eukaryotic cells.

Still further, a variety of transgenic organisms other than mice have been produced in which Tet repressor-transcriptional activator fusion proteins have been demonstrated to function as described in the instant specification. These include mosses (Zeidler et al. (1996) *Plant Mol. Biol.* 30: 199-205; attached as Appendix B); plants (*e.g.*, tobacco) (Weinmann et al. *Plant J.* (1994) 5(4); 559-569; Böhner et al. (1999) *Plant J.* 19(1): 87-95; attached as Appendices C and D, respectively); and insects (e.g., Drosophila) (Bello et al. (1998) *Devel.* 125: 2193-2202; Girard et al. (1998) *EMBO J.* 17(7): 2079-2085; Bieschke et al. (1998) *Mol. Gen. Genet.* 258(6): 571-579; attached as Appendices E, F and G, respectively). The fact that Tet repressor-transcriptional activator fusion proteins have been **demonstrated** to regulate target gene expression in a tetracycline-controlled fashion in a **variety of cell types and organisms** when constructed and used in accordance with the teachings of the instant specification indicates that the specification fully enables the construction and use of the Tet repressor-

inhibitor fusion proteins in a variety of transgenic organisms beyond just the exemplified transgenic mice.

Furthermore, at the time the invention was made, the teachings of the specification with regard to the general construction of transgenic organisms (see *e.g.*, pages 17-19), had been utilized by many groups in the production of a variety of transgenic organisms, including rats, pigs, sheep, and cows. Applicants refer the Examiner to:

Hammer et al. (*J. Anim. Sci.* 63(1): 269-278, 1986; attached as Appendix H), which teaches the production by microinjection of transgenic rabbits, sheep and pigs expressing human growth hormone under control of the mouse metallothionein-I promoter;

Pursel et al. (*J. Reprod. Fertil. Suppl.* 41: 77-87, 1990; attached as Appendix I), which teaches the production by microinjection of transgenic pigs expressing bovine and human growth hormone under control of the mouse metallothionein-I promoter;

Rexroad et al. (*J. Anim. Sci.* 69: 2995, 1991; attached as Appendix J), which teaches the production by microinjection of transgenic sheep expressing bovine growth hormone or human growth hormone-releasing factor under the control of the mouse transferrin promoter or the mouse albumin promoter, respectively; and

Ebert et al. (*Bio/Technology* 9: 835, 1991; attached as Appendix K), which teaches the production by microinjection of transgenic goats expressing longer acting tissue plasminogen activator under the control of the murine whey acid promoter.

Each of these references demonstrates the efficacy of the methodologies taught in the instant specification in the production of transgenic animals. Thus, it is clear from the teachings of these references that the methodologies taught by Applicants are equally valid for the production of non-murine animals transgenic for the Tet repressor-transcriptional inhibitor fusion proteins of the invention.

In view of the above arguments, Applicants respectfully request that the Examiner reconsider and withdraw the rejection of claims 1-11 and 13-26 under 35 U.S.C. § 112, first paragraph.

Rejection of Claims 1-26 Under the Judicially Created Doctrine of Obviousness-Type Double-Patenting

Claims 1-26 have been rejected under the judicially created doctrine of obviousness-type double patenting over U.S. Patent No. 5,866,755. This judicially created doctrine is grounded on public policy so as to prevent the unjustified or improper timewise extension of the "right to exclude" granted by a patent and to prevent possible harassment by multiple assignees. *In re Goodman*, 11 F3d 1046. In the case of the instant rejection under this doctrine, U.S. Patent No. 5,866,755 issued February 2, 1999 and has a pre-GATT filing date. Accordingly, this patent receives a patent term of 17 year from issuance and thus is scheduled to expire February 2, 2016. The instant application has a post-GATT filing date and has an earliest effective filing date of June 14, 1993. Accordingly, this application, upon issuance, would receive a patent term of 20 years from filing and thus would be scheduled to expire June 14, 2013. Furthermore, the '755 patent and the instant application are commonly owned. In view of the foregoing, Applicants submit that a rejection of the instant application under the judicially created doctrine of obviousness-type double patenting over U.S. Patent No. 5,866,755 is improper and should be withdrawn.

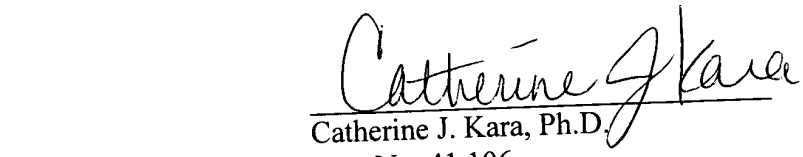
CONCLUSION

In view of the foregoing remarks, reconsideration of the rejections and allowance of all pending claims is respectfully requested.

If a telephone conversation with Applicants' attorney would expedite the prosecution of the above-identified application, the examiner is urged to call Applicants' attorney at (617) 227-7400.

Respectfully submitted,

LAHIVE & COCKFIELD, LLP



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APPENDIX A

1. A non-human transgenic organism having a transgene comprising a polynucleotide sequence encoding a fusion protein which inhibits transcription in eukaryotic cells, the fusion protein comprising a first polypeptide which is a Tet repressor or mutated Tet repressor that binds to a *tet* operator sequence, operatively linked to a heterologous second polypeptide which inhibits transcription in eukaryotic cells.

2. The organism of claim 1, wherein the first polypeptide of the fusion protein is a Tet repressor that binds to *tet* operator sequences in the absence but not the presence of tetracycline or a tetracycline analogue.

4. The organism of claim 2, wherein the first polypeptide comprises an amino acid sequence shown in SEQ ID NO: 17.

5. The organism of claim 1, wherein the first polypeptide of the fusion protein is a mutated Tet repressor that binds to *tet* operator sequences in the presence but not the absence of tetracycline or a tetracycline analogue.

7. The organism of claim 5, wherein the mutated Tet repressor has at least one amino acid substitution compared to a wild-type Tet repressor.

8. The organism of claim 7, wherein the mutated Tet repressor has an amino acid substitution at at least one amino acid position corresponding to an amino acid position selected from the group consisting of position 71, position 95, position 101 and position 102 of a wild-type Tn10-derived Tet repressor amino acid sequence.

9. The organism of claim 8, wherein the mutated Tet repressor comprises an amino acid sequence shown in SEQ ID NO: 19.

10. The organism of claim 1, wherein the second polypeptide comprises a transcription silencer domain of a protein selected from the group consisting of v-erbA, the Drosophila Krueppel protein, the retinoic acid receptor alpha, the thyroid hormone receptor alpha, the yeast Ssn6/Tup1 protein complex, the Drosophila protein even-skipped, SIR1, NeP1, the Drosophila dorsal protein, TSF3, SFI, the Drosophila

hunchback protein, the Drosophila knirps protein, WT1, Oct-2.1, the Drosophila engrailed protein, E4BP4 and ZF5.

11. The organism of claim 1, further having a second transgene comprising a gene of interest operably linked to at least one *tet* operator sequence.

12. The organism of claim 1, which is a plant.

13. The organism of claim 1, which is selected from a group consisting of a cow, a goat, a sheep and a pig.

14. A method for modulating transcription of the second transgene in the transgenic organism of claim 11, comprising administering tetracycline or a tetracycline analogue to the organism.

15. A non-human transgenic organism having a transgene comprising a polynucleotide sequence encoding a fusion protein which inhibits transcription in eukaryotic cells, the fusion protein comprising a first polypeptide which is a Tet repressor or a mutated Tet repressor that binds to a *tet* operator sequence, operatively linked to a heterologous second polypeptide which inhibits transcription in eukaryotic cells, wherein the transgene is integrated by at a predetermined location within a chromosome within cells of the organism.

16. The organism of claim 15, wherein the first polypeptide of the fusion protein is a Tet repressor that binds to *tet* operator sequences in the absence but not the presence of tetracycline or a tetracycline analogue.

18. The organism of claim 17, wherein the first polypeptide comprises an amino acid sequence shown in SEQ ID NO: 17.

19. The organism of claim 17, wherein the first polypeptide of the fusion protein is a mutated Tet repressor that binds to *tet* operator sequences in the presence but not the absence of tetracycline or a tetracycline analogue.

21. The organism of claim 20, wherein the mutated Tet repressor has at least one amino acid substitution compared to a wild-type Tet repressor.

22. The organism of claim 21, wherein the mutated Tet repressor has an amino acid substitution at at least one amino acid position corresponding to an amino acid position selected from the group consisting of position 71, position 95, position 101 and position 102 of a wild-type Tn10-derived Tet repressor amino acid sequence.

23. The organism of claim 22, wherein the mutated Tet repressor comprises an amino acid sequence shown in SEQ ID NO: 19.

24. The organism of claim 15, wherein the second polypeptide comprises a transcription silencer domain of a protein selected from the group consisting of v-erbA, the Drosophila Krueppel protein, the retinoic acid receptor alpha, the thyroid hormone receptor alpha, the yeast Ssn6/Tup1 protein complex, the Drosophila protein even-skipped, SIR1, NeP1, the Drosophila dorsal protein, TSF3, SFI, the Drosophila hunchback protein, the Drosophila knirps protein, WT1, Oct-2.1, the Drosophila engrailed protein, E4BP4 and ZF5.

25. The organism of claim 15, further having a second transgene comprising a gene of interest operably linked to at least one *tet* operator sequence.

26. A method for modulating transcription of the second transgene in the transgenic organism of claim 25, comprising administering tetracycline or a tetracycline analogue to the organism.

27. A transgenic organism having a transgene integrated into the genome of the organism and also having a *tet* operator-linked gene in the genome of the organism, wherein:

the transgene comprises a transcriptional regulatory element functional in cells of the organism operatively linked to a polynucleotide sequence encoding a fusion protein which inhibits transcription of said *tet* operator linked gene,

said fusion protein comprises a first polypeptide that is a Tet repressor operably linked to a heterologous second polypeptide which inhibits transcription of said *tet* operator-linked gene in eucaryotic cells,

said *tet* operator-linked gene confers a detectable and functional phenotype on the organism when expressed in cells of the organism,

said transgene is expressed in cells of the organism at a level sufficient to produce amounts of said fusion protein that are sufficient to inhibit transcription of the *tet* operator-linked gene; and

in the absence of tetracycline or a tetracycline analogue in the organism, said fusion protein binds to the *tet* operator-linked gene and inhibits transcription of the *tet* operator linked gene, wherein the level of expression of the *tet* operator-linked gene can be upregulated by administering tetracycline or a tetracycline analogue to the organism.

28. A transgenic organism having a transgene integrated into the genome of the organism and also having a *tet* operator-linked gene in the genome of the organism, wherein:

the transgene comprises a transcriptional regulatory element functional in cells of the organism operatively linked to a polynucleotide sequence encoding a fusion protein which inhibits transcription of said *tet* operator linked gene,

said fusion protein comprises a first polypeptide that is a mutated Tet repressor that binds to *tet* operator sequences in the presence, but not the absence, of tetracycline or a tetracycline analogue, operably linked to a heterologous second polypeptide which inhibits transcription of said *tet* operator-linked gene in eucaryotic cells,

said *tet* operator-linked gene confers a detectable and functional phenotype on the organism when expressed in cells of the organism,

said transgene is expressed in cells of the organism at a level sufficient to produce amounts of said fusion protein that are sufficient to inhibit transcription of the *tet* operator-linked gene; and

in the presence of tetracycline or a tetracycline analogue in the organism, said fusion protein binds to the *tet* operator-linked gene and inhibits transcription of the *tet* operator linked gene, wherein the level of expression of the *tet* operator-linked gene can be upregulated by depleting tetracycline or a tetracycline analogue from the organism.

APPENDIX B

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Short communication

Tetracycline-regulated reporter gene expression in the moss *Physcomitrella patens*

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Abstract

As ancestors of higher plants, mosses offer advantages as simple model organisms in studying complex processes such as development and signal transduction. Overexpression of transgenes after genetic transformation is a powerful technique in such studies. To establish a controllable expression system for this experimental approach we expressed a chimeric protein consisting of the *Tn10*-encoded Tet repressor and the activation domain of *Herpes simplex* virion protein 16 in the moss *Physcomitrella patens*. We showed that this protein activates transcription from a suitable target promoter (Top10) containing seven operators upstream of a TATA box. In media containing very low levels of tetracycline (1 mg/l), expression levels of a β-glucuronidase (GUS) reporter gene dropped to $< 1\%$ of that in the absence of tetracycline. This regulation is due to interference of tetracycline with the DNA binding activity of the Tet repressor portion of the chimeric transcriptional activator. Stable transformants grown for three weeks on tetracycline-containing media showed negligible GUS activity, whereas GUS was expressed strongly within 24 h of transfer to tetracycline-free media. Potent and stringently regulated expression of other, physiologically active genes is thus readily available in the moss system using the convenient Top10 expression system.

Mosses, situated at the base of higher-plant evolution, are useful objects for research into physiology and development, their simple nature providing insights into the complex biology of higher plants [see 6, 4, 21, 18]. The haploid gametophyte filaments can be grown indefinitely in sterile culture and easily observed at the cellular level [17, 5, 15, 12]. Cells are transformable via PEG- or particle-mediated DNA transfer [25, 23] and regenerate rapidly; stable transformants can be analysed within 2 to 3 weeks. The expression of introduced genes in mosses can give information regarding gene function (reverse genetics) [17, 25, 19].

Overexpression studies with constitutive promoters are limited by the absence of strict controls for unexpected epistatic effects of the transformation procedure. Although widely assumed, lines transformed with 'empty' vectors are not necessarily appropriate controls, as they are not genetically identical to the overexpressors. Moreover, overexpression of certain gene products can

reduce viability and interfere with the regeneration process. Various inducible plant promoters, including those regulated by light [10] heat shock [26], nitrate [1], or even phytohormones [7, 16] could in principle be used to control expression of transgenes. Each of these inducing treatments has, however, dramatic and pleiotropic physiological consequences for plants, irrespective of any transgenes.

Inducible systems based on the Tet repressor (TetR) of *Escherichia coli* have been developed for use in eukaryotes [8, 9, 22]. In plants expressing high levels of TetR the activity of a cauliflower mosaic virus (CaMV) 35S promoter derivative containing three operators in the vicinity of the TATA box is repressed at least 100-fold. Tetracycline penetrates living cells readily [9]. As the affinity of the repressor for Tc is high [27], very low levels of exogenous Tc (1 mg/l) are sufficient for dissociation of the repressor from the promoter *in planta*. Such low levels of Tc have negligible physiological impact [8, 9, 28].

The Tc-inducible system requires a strong promoter driving TetR expression because high levels of repressor protein are needed for efficient displacement of the transcriptional initiation complex. This potential problem has been circumvented by fusing TetR to the VP16 transcriptional activation domain from *Herpes simplex*, thereby turning the repressor into an activator, tTA [11, 28]. As illustrated in Fig. 1, the target promoter, Top10, contains several operators upstream of a TATA-box. In the absence of Tc, tTA binds to the operators, thereby bringing the VP16 activation domain to the promoter region, which leads to transcriptional activation.

As there is no direct competition for the binding sites with eukaryotic transcription factors, and because less than 100% occupancy of the operators are sufficient for activation, less tTA is needed for activation than TetR for repression. In the presence of Tc, promoter activity is abolished due to dissociation of tTA. In plants, background levels in the inactive state are lower than when TetR has been used as a *bona fide* repressor. The Top10 system has the advantages of requiring only a weak constitutive promoter to drive tTA and showing stringent down-regulation and strong activation. Although Top10 transformants must be continually supplied with tetracycline to prevent expression, they have the advantage of allowing induction on normal (– Tc) media and so can be compared with the wild type under identical conditions. In our moss system, protonemata are grown on agar plates or in liquid medium, so the provision or removal of Tc is straightforward.

We constructed a Top10-based GUS reporter

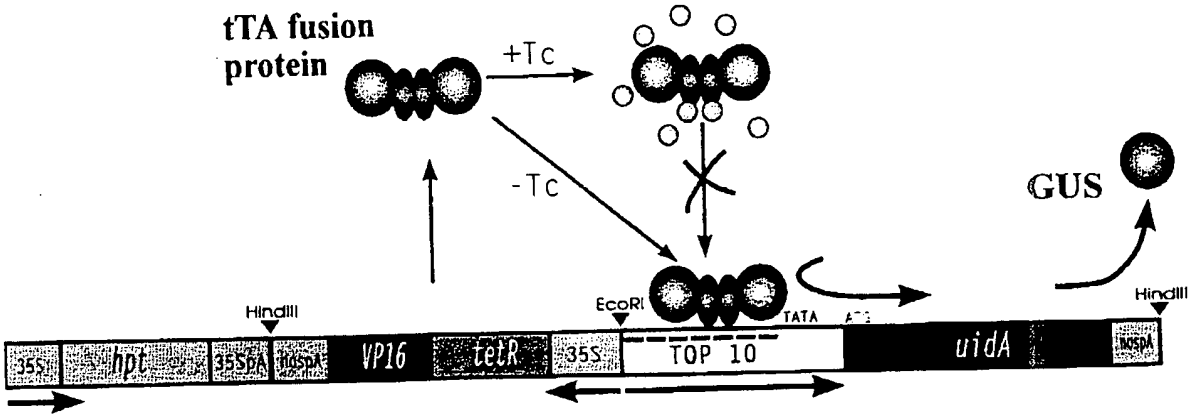


Fig. 1. Function of the Top10 system. The chimeric molecule, tTA, a fusion of the *E. coli* TetR molecule and the *Herpes simplex* virus activator VP16, binds to the Top10 promoter and initiates transcription. In the presence of Tc, however, tTA binding to the promoter is impeded, abolishing gene expression [28]. (*nos pA*, polyadenylation signal of the *nos* gene; *uidA*, β-glucuronidase gene of *E. coli*; *35S pA*, polyadenylation signal of CaMV 35S gene).

plasmid (pGLtVT, Fig. 1) bearing a hygromycin-resistence gene for stable transformation of moss protoplasts. The source plasmids utilized are summarised in Table 1. The *Hind* III-*Eco* RI 35S-VP16-*tet* R-*nos* PA cassette was removed from ptetVP16. A similar restriction fragment containing the Top10-*uidA-nospA* cassette was removed from pTop10. These cassettes were ligated together into the *Hind* III site of pGL2, oriented so that no read-through was possible, to give pGLtVT (detailed description of this plasmid is available upon request). The plasmid was transformed into *Physcomitrella* protoplasts essentially as described [25].

Quantitative estimations of GUS activity based on MUG (4-methylumbelliferyl-β-D-galactoside) assay [14] are shown in Fig. 2. For each assay 5 μg total protein extract was used and incubation of the 100 μl reaction mix at 37 °C was stopped at different time points. Endogenous GUS activities of *Physcomitrella* protonemal or protoplast extracts in MUG assay were very low, <2 nmol MU min^{-1} mg^{-1}. Accordingly, even after several days incubation with X-Gluc at 500 μg/ml [13] no *in situ* staining was apparent. Transient GUS expression with pBluescript or pTop10 plasmid controls gave similarly low backgrounds. Of the numerous 35S-promoter-driven GUS constructs tested, only the pNco-GUS plasmid gave useful expression (>500 nmol MU min^{-1} mg^{-1} in transient assay; Fig. 2a). This value is nevertheless much lower than that of typical 35S-GUS-transformed dicotyledons (ca. 3000–30000 nmol MU min^{-1} mg^{-1} [13 and data not shown]. Similar results with 35S constructs have been obtained in other laboratories using PEG-mediated DNA transfer [24]. The strongest naturally occurring promoter in transient assay was that of the rice *Actin1* gene [29]. Potentially significant in this context is that pActin1 is active in gametophytic pollen grains as well as in the sporophyte [29]. The heat-shock-inducible promoter from soybean (pHS-GUS), active in higher plants, was not effective in moss; transient assays before or 24 h after five 30 s heat treatments at 45 °C, a non-lethal treatment, showed no significant GUS activity (Fig. 2a).

As shown in Fig. 2, in the absence of Tc the 35S-driven tTA-Top10-GUS construct pGLtVT gave 60% stronger transient activity than pNco-GUS. Tc added to the recovery medium after transformation reduced expression from pGLtVT to background levels (2 nmol MU min^{-1} mg^{-1}). As this plasmid carries a *hpt* hygromycin-

Table 1. Construct descriptions.

Construct	Description	Source
pBluescript	Commercial cloning vector	Stratagene
pNco-GUS	*E. coli uidA* gene cloned as a *Sal* I-*Nco* I fragment in pDH51	[3, 20]
pHS-GUS	Soybean heat shock gene promoter (*Gmhsp*) fused transcriptionally to *E. coli uidA* in pBIN19	[26, 2]
pActin1-GUS	Rice Actin1 promoter transcriptionally fused to *E. coli uidA* with *nos* poly(A) signal in pBluescript	[29]
pGL2	*hpt* gene cloned in *Bam* HI site of pDH51	[20]
pTop10	Top10 controlled *E. coli uidA* gene with *nos* poly(A) signal; cloned as an *Eco RI-Hind* III fragment in pUC19	[28]
ptetVP16	CAMV 35S-controlled *E. coli TetR* translationally fused to *H. simplex* virus *VP16* (tTA); cloned as an *Eco* RI-*Hind* III fragment in pUC19	[28]
pGLtVT	pTop10 *Eco* RI-*Hind* III cassette and ptetVP16 *Eco* RI-*Hind* III cassette cloned together in *Hind* III of pGL2	This paper, [20, 28]

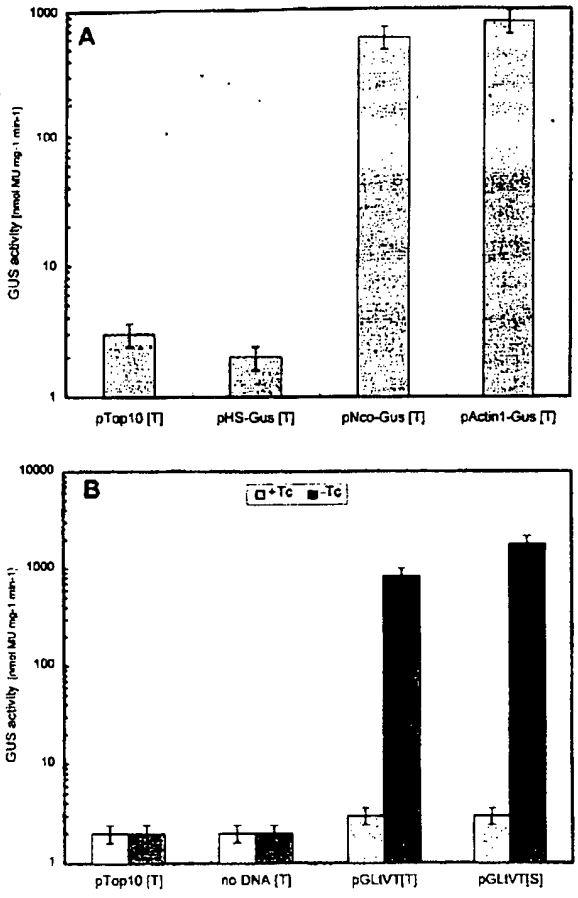


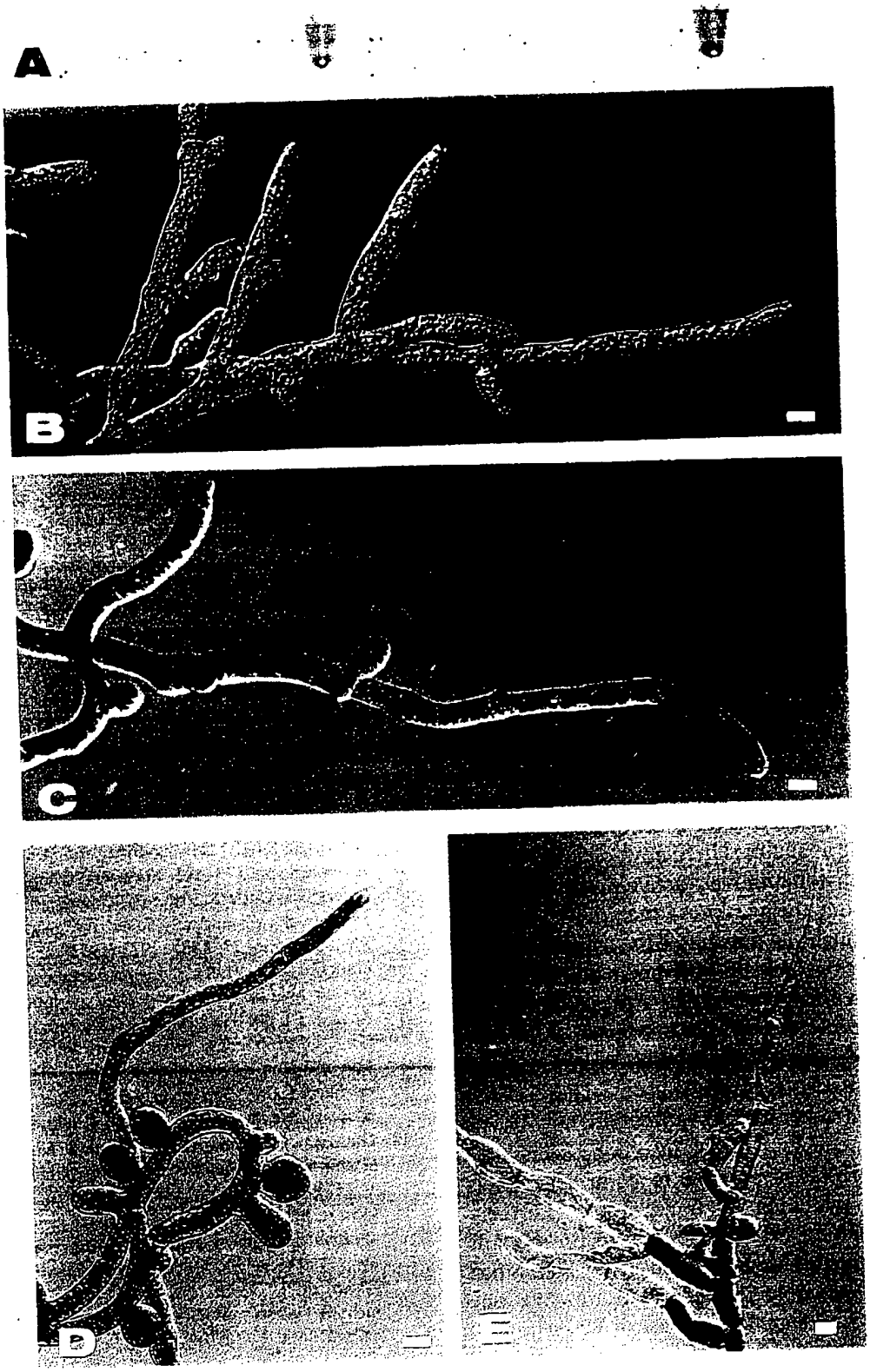
Fig. 2. A. GUS activity of different constructs. B. GUS activity of different constructs with (light bars) and without Tc (dark bars) in the culture medium; [T], transient assay; [S], stable transformants. Spontaneous MUG hydrolysis rate (derived from a control reaction without extract) was subtracted from all measurements. The error bars represent the standard deviation based on 3 independent measurements.

resistance gene it was possible to select stable transformants and study the Top10 system in these. Such transformants subcultured for several weeks alternately on selective (30mg/l hygromycin) and non-selective media in the presence of Tc, were finally transferred to – Tc media for 3 days prior to MUG assay. The exracts showed three-fold higher GUS levels than with transient pNco-GUS. Material kept on + Tc showed background activities (Fig. 2b). All of the moss lines which were selected with the above mentioned procedure were able to express GUS if kept on – Tc.

These quantitative assays were consistent with X-Gluc *in situ* staining results (Fig. 3). Transient expression in protoplasts was clearly and strongly Tc-dependent (Fig. 3a). Stably transformed clones on + Tc showed insignificant staining (Fig. 3b), although filaments which had grown away from the medium occasionally showed faint blue coloration after prolonged incubation. In contrast, filaments grown for 3 days on – Tc stained intensely (Fig. 3c). Some cells, produced under these conditions, were morphologically abnormal, showing a bulbous rather than a tubular form, possibly due to deletrious effects of GUS expression. Induction of the reporter after transfer from + Tc to – Tc for 3 days was seen in all cells (Fig. 3d). This strong, rather uniform staining was in contrast to the results obtained with the reverse treatment, – Tc to + Tc. After 3 days on + Tc strong GUS activity was apparent only in cells which had developed under – Tc conditions; cells which arose under + Tc were weakly stained or, towards the growing filament tips, not stained at all (Fig. 3e). This is to be expected as GUS is a particularly stable enzyme [13]; most of the activity produced under – Tc would remain even several days after GUS expression was shut off. A certain amount of cytoplasmic carryover would also be expected at cell division.

Moss filaments can be grown in liquid culture in the presence of Tc, so that uniform inactivation can be maintained indefinitely. Induction is convenient because Tc can be removed simply by passing the culture through a sieve and resuspending in Tc-free medium. Induction is rapid, strong X-Gluc-staining being seen even after only

Fig. 3. A. Transient GUS assay, from left to right: double transformation of activator and reporter plasmid (30 and 20 μg DNA, respectively) with and without Tc; transformation with pGLtVT, with and without Tc. B–E. Stable transformants. Cultured for two weeks without (B) and with (C) Tc. Filaments grown on Tc for two weeks then transferred to – Tc media for 3 days (D). Filaments grown on – Tc media for two weeks and then transferred for 3 days to media containing Tc (E). The bar represents 10 μm.


A
B
C
D

24 h on −Tc. Although the speed of down-regulation following addition of Tc is likely to be very rapid on account of the binding avidity of TetR and the speed of penetration into the cells [27, 28], the stability of the GUS enzyme prevented our measuring this (Fig. 3e). The 35S and Top10 promoters were orientated in opposite direction to prevent readthrough effects, but an enhancement effect of the 35S promoter on Top10 cannot be ruled out. On the other hand, the data we obtained from MUG assay and *in situ* staining imply very stringent regulation.

On a protein basis, the GUS activities of transgenic dicotyledon tissues are typically 10- to 100-fold higher than those obtained here [13]. The Top10-GUS reporter presumably reflects the activity of the *35S* promoter driving tTA under −Tc conditions, hence stronger Top10 expression might be achievable using a stronger promoter to drive the *TetR-VP16*. Meaningful though the specific activities are, the strength of expression on a cell basis is clearly apparent from *in situ* staining (Fig. 3).

In this work we were able to demonstrate switchable expression in the moss system. The tTA activator is obviously effective in this background. By re-engineering the construct to allow insertion of coding sequences other than GUS downstream of Top10 (work in progress), a powerful physiological tool for overexpression studies will become available. Up to now, overexpression studies with constitutively-active promoters have suffered from the weakness that the transgenic organism might as a result of the transformation procedure be genetically aberrant in other aspects, and show a phenotype related to these rather than to overexpression of the transgene. Although widely used, transformation with 'empty' vectors is hardly an adequate negative control here, as the insert itself (rather than its expression) might have significant effects (site of insertion, silencing). Using Top10 it is possible to control for such effects by switching off transcription. Furthermore, the levels of Tc necessary to do this are orders of magnitude below those at which other Tc-related physiological effects occur. We observed the first significant effect of Tc on protoplast regeneration and cell growth at concentrations 100-fold higher than that used for effective down-regulation of Top10. Thus +Tc-treated transformants can be compared directly to the wildtype under +Tc conditions. In this respect Top10 is superior to other potential inducible systems such as those based on heat-shock or phytohormone effects. The tight regulation shown by the expression system allows titration of Top10 expression with very low amounts of Tc.

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TECHNICAL ADVANCE

APPENDIX C

A chimeric transactivator allows tetracycline-responsive gene expression in whole plants

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Summary

The chimeric transcriptional activator tTA, a fusion between the Tn*10* encoded Tet repressor and the activation domain of the *Herpes simplex* virion protein VP16, was stably expressed in transgenic tobacco plants. It stimulates transcription of the β-glucuronidase (*gus*) gene from an artificial promoter consisting of 7 *tet* operators and a TATA-box. Tetracycline, which interferes with binding of tTA to operator DNA, reduces *gus* expression over several orders of magnitude. This stringency of regulation suggests that the system can be used to construct transgenic plants encoding a potentially lethal gene product. Furthermore, the specific and fast inactivation of tTA allows study of the stability of RNAs and proteins.

Introduction

Expression of foreign genes in transgenic plants is a widely used tool to confer new characters to different species (Schell, 1987; Willmitzer, 1988). In addition, enhancing or reducing the expression of endogenous genes helps us to understand the contribution of a defined gene product to the phenotype. The ability to control expression of a gene via a highly specific mechanism offers unique opportunities to study the physiological functions of certain gene products at different stages of development. The correlation of the phenotype with the kinetics of induction allows differentiation between primary and secondary consequences, which generates another advantage of a regulated expression system. Moreover, a stringently regulated promoter is absolutely required, if the expression of a gene product of interest interferes with the regeneration process.

Ideally, an inducible promoter should show extremely low or no basal levels of expression in the absence of inducing conditions, a high level of expression in the induced stage, and an induction scheme that does not otherwise alter the physiology of the plant. The last requirement, especially, renders the use of endogenous promoters, that respond to stimuli like heat (Schöffl *et al.*, 1989), wounding (Keil *et al.*, 1989), nitrate (Back *et al.*, 1991) or light (Gilmartin *et al.*, 1990) less favorable. A more promising approach is to combine regulatory control elements from other organisms, that respond to signals usually not encountered by a plant, with the general plant transcription machinery.

Based on this idea, two different concepts of gene control can be realized, i.e. promoter-repressing systems and promoter-activating systems. One way to construct a promoter repressing system is to use bacterial repressors to compete directly with plant transcription factors and/or RNA polymerases for binding (Gatz *et al.*, 1992; Wilde *et al.*, 1992). Using the Tn*10* encoded Tet repressor (TetR) in combination with a suitably engineered Cauliflower Mosaic Virus (CaMV) 35S promoter with three integrated *tet* operator sites we have succeeded in constructing a tightly repressible expression system (Gatz *et al.*, 1992). The DNA-binding affinity of TetR can be abolished by low amounts of tetracycline (Tc). The high equilibrium association constant of about 10^{-9}M for the repressor–inducer complex (Takahashi *et al.*, 1986) ensures efficient induction at Tc concentrations that do not even inhibit the growth of procaryotes (Geissendörfer and Hillen, 1990). Addition of Tc leads to a 200- to 500-fold induction of promoter activity throughout intact tobacco plants without any obvious inhibition of plant growth (Roeder *et al.*, 1994).

In this paper we describe the characterization of a Tc-dependent expression system in transgenic plants that combines the features of TetR with those of a promoter-activating system. Because of the modular organization of transcription factors (Frankel and Kim, 1991), eucaryotic activation domains can be fused to procaryotic repressor proteins thus turning them into transcriptional activators (Brent and Ptashne, 1985; Labow *et al.*, 1990). Fusion of

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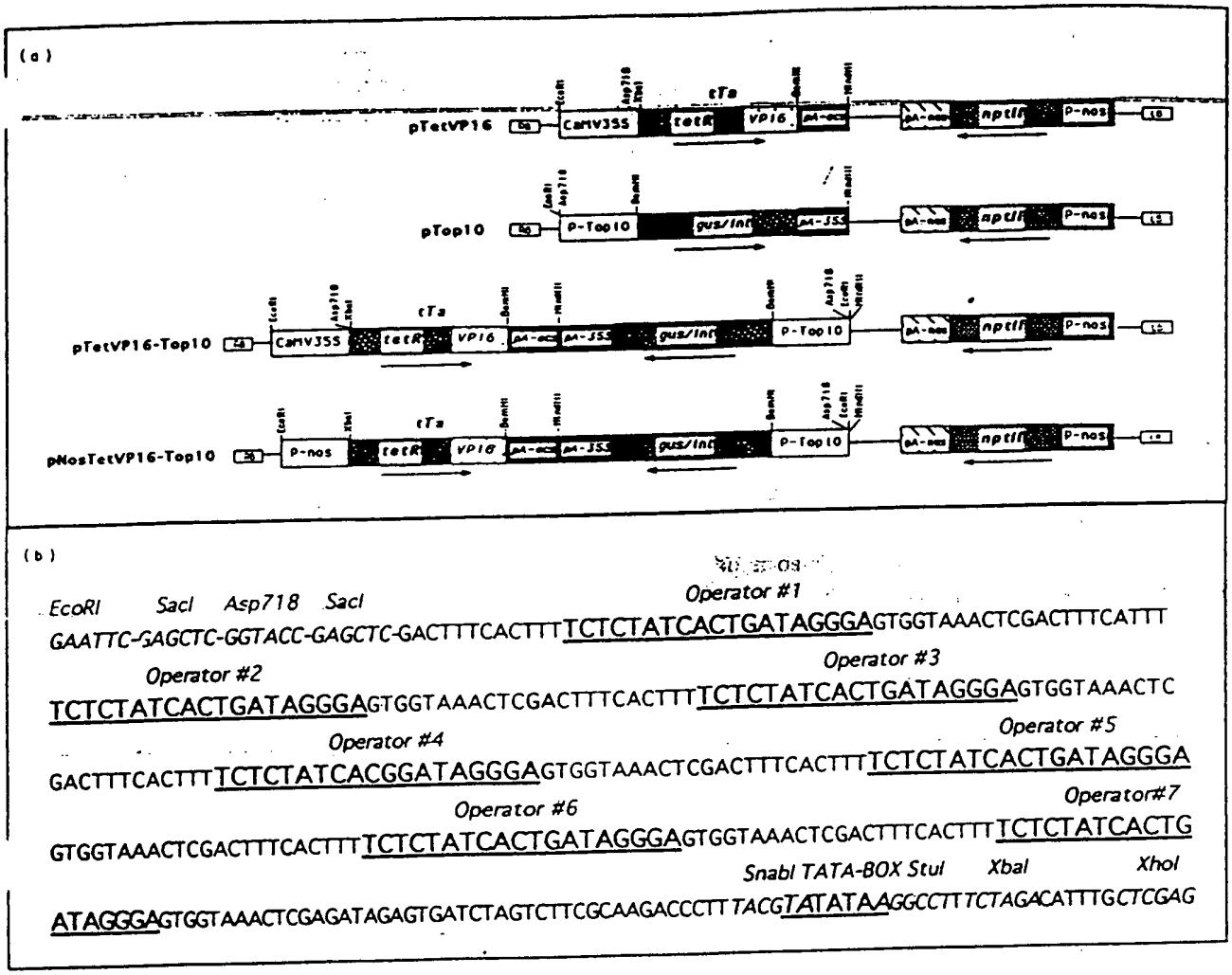

Figure 2. Constructs.

(a) Diagram of the chimeric genes used for the establishment of a Tc-responsive promoter in transgenic plants. pTetVp16 contains the transcriptional transactivator gene (*tTA*) under the control of the CaMV 35S promoter. pTop10 encodes the *gus/int* gene under the control of the chimeric promoter consisting of seven *tet* operator sites 5' to a TATA-box (TATATAA). pTetVP16-Top10 contains both chimeric genes on one T-DNA. pNosTetVP16-Top10 differs from pTetVP16 with regard to the promoter (P-Nos) driving *tTA* expression. RB, right border; LB, left border; pA, polyadenylation signal. P—promoter; *nptII*, neomycinphospho-transferase; *ocs*, octopine synthase; *nos*, nopaline synthase. *gus/int*: β-glucuronidase with an intron (Vancanneyt *et al.*; 1990). Arrows indicate the transcriptional orientation.

(b) Sequence of the target promoter P-Top10. The sequences of the operators and the TATA-box are underlined, recognition sequences for restriction sites are written in italics.

Activity of the target promoter P-Top10 during the regeneration process

In order to characterize the function of tTA and its target promoter in stably transformed plants we cloned the respective chimeric genes on binary vectors which were designed for *Agrobacterium tumefaciens*-mediated gene transfer (Bevan, 1984). As a first step we replaced the *gus* gene of the reporter construct by a modified version that contains an intron (Vancanneyt *et al.*, 1990). Thus Gus activity of transformed plant tissue can be monitored very early after the co-cultivation step, because the gene is not properly expressed in *Agrobacterium*. Four different transgenic tobacco lines were generated. Figure 2 depicts the different chimeric genes that were cloned into BIN19. pTetVP16 encodes only the 'activator construct' whereas pTop10 carries only the 'target construct' adjacent to the chimeric kanamycin-resistance gene. pTetVP16-Top10 contains both constructs on the same T-DNA. To avoid a potential influence of the CaMV 35S enhancer on transcription from the target promoter P-Top10 the reporter construct was placed downstream of the chimeric *tTa* gene. A tail-to-tail orientation of both genes was chosen as a further precaution. pNosTetVP16-Top10 differs from pTetVP16-Top10 with regard to the promoter driving the expression of tTA: whereas pTetVP16-Top10 encodes

Table 1. Gus activities of nine highest expressing TetVP16-Top10 plants and a representative Triple-Op/gus plant (TX)

Transformant	pmol 4 MU min^{-1} mg^{-1} protein
5	820
6	1550
13	3190
22	1780
24	1060
30	1470
42	2045
43	6070
47	7530
TX	13 000

Gus activity was determined using the fluorometric assay of Jefferson (Jefferson *et al.*, 1987). One leaf was taken when shoots had just formed small roots on kanamycin containing medium.

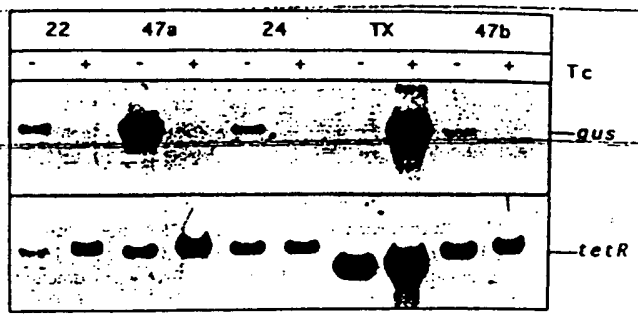

Figure 5. Northern blot analysis of TetVP16-Top10 plants.
RNA was prepared either directly from plants grown under axenic conditions on 2MS medium (lanes marked –) or after infiltration with 1 mg l^{-1} Tc and 48 h incubation on Tc-containing medium (lanes marked +). In lanes TX+ and TX– RNA of a transgenic plant containing the *gus* gene under the control of the Tc-inducible TX-promoter was loaded to demonstrate the reciprocal effect of Tc. The blot was first probed with a restriction fragment containing the *gus* coding region, and subsequently with a 695 bp tetR fragment.

The average Gus activity was calculated to be 660 pmol 4 MU min^{-1} mg^{-1} protein (U). Expression levels of the nine highest expressing plants varied between 7530 and 820 U (Table 1). These plants were kept for further analysis. Extracts from Top10 transformants and NosTetVP16-Top10 transformants were incubated for 12 h in order to detect even low amounts of activity. Under these conditions only four Top10 plants showed a Gus activity of ca. 50 U. Activities in all the other shoots were indistinguishable from activities measured in untransformed control plants (10 U). To assess the effect of Tc on Gus activity of TetVP16-Top10 plants, we first compared tissues which were newly formed either in the presence or absence of Tc. Thus, we avoided potential complications due to the stability of the Gus protein. The upper panel of Figure 4 shows roots from plants nos 22, no. 24 and 30; the lower panel shows regenerating shoots from explants taken from plant no. 47 and from a transgenic plant expressing the *gus* gene under the control of the Triple-Op promoter (TX) which is negatively regulated by TetR (Gatz *et al.*, 1992). Even after staining overnight no detectable Gus signal was observed in roots grown in 2MS medium with 1 mg l^{-1} Tc indicating a very stringent regulation. Quantitation using the fluorometric assay (Jefferson *et al.*, 1987) revealed that tissue formed in the presence of Tc (roots, callus, regenerating shoots) never showed higher Gus activity than untransformed controls, even when the extracts were incubated for more than 12h. Under these conditions, the Triple-Op promoter, which is inducible by Tc, gives values between 100- and 250-fold regulation. As the Top10 promoter in the presence of Tc yields no activity above the background measured in untransformed control plants, we cannot calculate an equivalent numerical value for the efficiency of regulation. When shoots that had formed on Tc were placed on 2MS without Tc, Gus activity reaccumulated, which shows that the regulation is reversible.

The explants shown in the lower pannel of Figure 4 indicate that Gus expression levels are sensititive to Tc not only in newly formed shoots, but also in leaf explants, which had expressed Gus activity before being put on Tc. We confirmed this on the RNA level by Northern blot analysis of RNA from leaves being vacuum infiltrated with and without Tc (Figure 5). As expected, plants nos 22, 47 and 24 showed a Tc-mediated decrease in mRNA accumulation. Plant TX, which contains the *gus* gene under the control of the TetR-regulated promoter, shows the positive effect of Tc in that system. At the level of total RNA no background activity could be detected, with longer exposures showing only signals originating from cross-hybridization to ribosomal RNA. Whereas no. 47 showed the expected expression levels comparable with the levels given by the CaMV 35S promoter (lane 47a, see Table 1), plants nos 22 and 24 showed unexpectedly low mRNA levels. As the RNA was isolated 3 months after quantitation of Gus expression levels, it seems that the target promoter gets silenced when the plants grow older. This was confirmed by analyzing RNA of plant no. 47 1 year after the transformation. The decrease in the amount of expression is demonstrated in lane 47b (Figure 5). This instability of the activity of the target promoter was observed in all our transformants. Rehybridization of the Northern blot with the *tetR* probe revealed, that the silencing of the promoter was not due to reduced expression of *tTA* mRNA. Gus activities of 10 kanamycin-resistant seedlings from plants nos 13, 30 and 47 were determined to be 430 U, 50 U and 250 U, respectively, which still is significantly lower when compared with the expression levels of the young transformants (Table 1). All of them showed again stringent sensitivity to Tc.

levels of three plants transformed with pTetVP16-Top10, which showed different Gus expression levels (Table 1) were in the same range. The gel shift analysis provides biochemical evidence for tTA expression in transgenic plants (Figure 8b). Owing to the change in molecular weight and charge the complex has a reduced electrophoretic mobility as compared with the Tet repressor–operator complex.

Discussion

We have established a regulated promoter for transgenic plants whose activity is turned off in the presence of low amounts of Tc. Regulation is based on an activation mechanism: a chimeric protein (tTA) consisting of the Tn*10*-encoded Tet repressor (TetR) fused to the activation domain of a eucaryotic transcriptional activator (VP16) (Gossen and Bujard, 1992) was expressed in plants. When bound to an array of *tet* operator sequences upstream of the TATA-box of the CaMV 35S promoter it activates transcription *in vivo*. In the presence of Tc, which prevents tTA from binding to the operator sequences, the promoter is inactive. In contrast, the principle of our previously established Tc inducible system (Gatz *et al.*, 1992) is based on repression. The Tn*10*-encoded Tet repressor sterically interferes with the establishment of a functional transcription initiation complex if three operator sites are suitably engineered in the vicinity of the TATA-box of the CaMV 35S promoter. Addition of Tc induces gene expression because it prevents TetR from binding. For both expression systems the features of the Tn*10*-encoded TetR—namely its capacity to bind to operator DNA and the action of Tc to interfere with binding (Hillen *et al.*, 1984)—were exploited. This discussion includes a comparison of the promoter-activating versus the promoter-repressing system for the transcriptional regulation in plants.

Transient assays

Transient assays proved that tTA is functional in plants. This was expected in view of the observation that the acid domain of VP16, originally encoded by *herpes simplex* virus, can activate transcription in a variety of organisms like yeast (Berger *et al.*, 1992), mammalian cells (Sadowski *et al.*, 1988), insects (Wampler and Kadonaga, 1992) and plants (McCarty *et al.*, 1991). As in the other systems, we observe 'squelching' effects at higher tTA concentrations, which indicates that tTA interacts with some component of the transcriptional machinery, even if it is not bound to operator DNA (Berger *et al.*, 1990). At optimal concentrations, the activity of the tTA-driven promoter is higher than the activity mediated by the CaMV 35S promoter. However, its background activity in the absence of the activator was rather high (10–18% of the optimal tTA-dependent activity). It might well be that the sequence between the operators upstream of the TATA-box contains a cryptic activation site that is recognized in protoplasts. We have shown previously that the operator sequence itself does not mediate activation (Frohberg *et al.*, 1991). In addition, sequences between +1 and −50 in the same vector do not confer promoter activity (data not shown). Upon addition of Tc activity of the reporter construct dropped, similar to results obtained earlier in stably and transiently transformed Hela cells (Gossen and Bujard, 1992). However, at 2 mg l^{-1} Tc, a concentration which causes maximal induction in the repressed system, tTA is not completely inactivated. These results are in contrast to comparable transient expression experiments in mammalian cells, where even lower Tc concentrations completely inhibit tTA-dependent activation (Gossen and Bujard, unpublished results). As in stably transformed plants even 1 mg l^{-1} Tc is sufficient for complete inactivation of tTA (see below), we would assume, that the incomplete inactivation is not due to a reduced affinity of tTA for Tc. We would rather favor the explanation that at the high concentrations of transactivator and reporter DNA present in a transient expression system, the tTA–Tc complex might still cause some activation due to unspecific binding to the introduced target plasmid. In conclusion, these results indicate that the tTA- based system is not appropriate for the regulated expression in transient systems. In contrast, we do not find any background activity and maximal inducibility by Tc in transient assays when TetR is used to to repress transcription (Gatz *et al.*, 1992).

Stable transformants

The problems encountered in transient assays were not observed in stably transformed plants. Forty-six transgenic plants containing the Top10 construct did not show any detectable Gus activity, four plants showed low activity, probably due to the integration of the T-DNA in the proximity of enhancer elements that might interact with the TATA-element of the target promoter. If the chimeric *tTA* gene was introduced along with the target promoter, 46 out of 60 kanamycin-resistant plantlets expressed the *gus* gene if grown in the absence of Tc. The difference in the expression levels between the inactive state and the active state of this system is difficult to quantify because Gus activity in the presence of Tc is close to zero. Similar results were obtained in HeLa cells, where luciferase activity was less than 2 U in cells grown in the presence of Tc and up to 257 100 U in the absence of Tc resulting in a regulation factor of at least 1 × 105 (Gossen and Bujard, 1992). Gus activity in untransformed plants and TetVP16-Top10 plants grown in the presence of Tc was 10 U in our

very useful for the direct comparison of the stabilities of two proteins.

Expression of tTA in transgenic plants

The transient assays already indicated, that high levels of *tTA* expression might lead to the inhibition of transcription due to squelching effects. Consistently we found that transgenic plants synthesize less mRNA for tTA than for TetR (Figure 8a). The gel shift analysis (Figure 8b) allows a rough estimation of the difference between TetR and tTA expression levels: 0.12 µg of the extract derived from plant Tet1#2 encoding TetR retard about the same amount of operator encoding DNA as 6 µg of the TetVp16#17 extract, indicating that tobacco plants tolerate about 50-fold less tTA than TetR. However, this estimation is based on the assumption that tTA binds operator DNA with a similar affinity as TetR, and that it is as stable in the crude extract as TetR. The Nos promoter, which is reported to be 30-fold less active than the CaMV 35S promoter (Sánders *et al.*, 1987), is too weak to synthesize sufficient amounts of tTA in leaves. The blue staining we observed in callus of NosTetVP16-Top10 plants proves that the construct is functional. We have found no direct comparison of Nos promoter activity in leaves and callus in the literature. However, it has been described, that the Nos promoter is under developmental control (An *et al.*, 1988) as well as wound inducible and auxin inducible (An *et al.*, 1990). Therefore it is very likely, that its expression is higher in callus than in leaves. This indicates that tTA levels have to exceed a certain threshold to be able to drive expression from the target promoter.

In conclusion, we have established a Tc-dependent expression system in transgenic plants and demonstrated its potential to regulate efficiently expression of a transgene. For some applications, this system, which is negatively regulated by Tc, might be superior to the previously established Tc-inducible promoter (Gatz *et al.*, 1992): (i) it is particularly useful if the stringency of the regulation has to be very high; (ii) It can be used for the study of mRNA and protein decay rates.

Experimental procedures

Plants, bacterial strains and media

Nicotiana tabacum W38 was obtained through 'Vereinigte Saatzuchten' (Ebstorf, Germany). Plants in tissue culture were grown under a 16 h light/8 h dark regime on Murashige and Skoog medium (Murashige and Skoog, 1962) containing 2% sucrose (2 MS). *Escherichia coli* strain DH5α (Bethesda Research Laboratories, Gaithersburg) was cultivated using standard techniques (Sambrook *et al.*, 1989). *Agrobacterium tumefaciens* strain C58CX1 containing pGV2260 (Deblaere *et al.*, 1985) was cultivated in YEB medium (Vervliet *et al.*, 1975).

Reagents

DNA restriction and modification enzymes were obtained from Boehringer Mannheim (Ingelheim, Germany) and New England Biolabs (Danvers, USA). Chemicals were obtained through Sigma Chemical Co. (St. Louis, USA) or Merck (Darmstadt, Germany).

Recombinant DNA techniques

Standard procedures were used for recombinant DNA work (Sambrook *et al.*, 1989).

Constructs

A diagram of the final constructs is shown in Figure 2. pTetVP16: pTet1 (Gatz *et al.*, 1991), which contains the *tetR* coding region between the CaMV 35S promoter and the octopine synthase (*ocs*) transcriptional terminator, was digested with *Bam*HI and *Sph*I and treated with T4-Polymerase to generate blunt ends. Religation of the vector resulted in the loss of the *Xba*I, *Sal*I, *Pst*I and *Sph*I sites between the coding region and the *ocs* transcriptional terminator. The resulting plasmid pTet1Δ was digested with *Xba*I and *Bam*HI in order to excise *tetR* sequences downstream from amino acid 3. The coding sequence of tTA was gained by *Xba*I/*Bam*HI digestion of pUHD 15-1 (Gossen and Bujard, 1992) and ligated into pTet1Δ cut with *Xba*I and *Bam*HI to yield pTetVP16. pUC-TetVP16: The *tTA* gene under the control of the CaMV 35S promoter and the *ocs* transcriptional terminator was excised from pTetVP16 as an *Eco*RI/*Hin*dIII fragment and inserted into pUC18 cut with *Eco*RI and *Hin*dIII (Figure 1). pUC-Top10: pUC-Top10 was derived from pIGF107 (Gatz *et al.*, 1991), which contains the coding region of the bacterial *chloramphenicol acetyl transferase* (*cat*) gene under the control of a modified CaMV 35S promoter. This promoter contains a number of additional unique restriction sites between positions −1 and −53. The plasmid was linearized with *Spe*I at position −53, the protruding ends were filled in using Klenow DNA polymerase, and a synthetic 55 bp oligonucleotide (Gatz *et al.*, 1991) encoding two operators and a *Bgl*II site was inserted. The promoter fragment was cloned as an *Eco*RI/*Xho*I (fill-in) fragment into pGus (Köster-Töpfer *et al.*, 1989) cut with *Eco*RI and *Sma*I. The resulting plasmid pSpe-Gus was cut with *Asp*718 and *Bgl*II(fill-in) and ligated with the *Asp*718/*Xho*I (fill-in) fragment from pUHC 13-4 (Gossen and Bujard, 1992), so that the CaMV 35S enhancer was replaced by seven *tet* operators. In the course of this step part of the 55 bp oligonucleotide was unintentionally deleted, so that seven *tet* operator sequences (instead of nine) were left. The sequence of this promoter (P-Top10) is shown in Figure 2(b). pUC-Top10-Gus/int: the coding region of the β-glucuronidase (*gus*) gene and the nopaline synthase (*nos*) polyadenylation signal from pUC-Top10 was excised with *Bam*HI and *Hin*dIII and replaced by the coding region of a modified *gus* gene containing an intron (Vancanneyt *et al.*, 1990) and the CaMV 35S polyadenylation signal. pTop10: The *gus/int* gene under the control of the synthetic Top10 promoter was cloned as an *Eco*RI/*Hin*dIII fragment into pBIN19 (Bevan, 1984) cut with *Eco*RI and *Hin*dIII. pTetVP16-Top10: a *Hin*dIII linker was cloned into the *Eco*RI (fill-in) site of pUC-Top10-Gus/int. The *gus/int* gene under the control of the synthetic Top10 promoter was cloned as a *Hin*dIII fragment into pTetVP16 *Hin*dIII. For further experiments we chose a plasmid that contained the *tTA* gene and the *gus* gene tail to tail (Figure 2). pNosTetVP16: pTetVP16 was cut with *Eco*RI and *Asp*718 (fill-in).

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APPENDIX D

TECHNICAL ADVANCE

Transcriptional activator TGV mediates dexamethasone-inducible and tetracycline-inactivatable gene expression

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Summary

A chemically regulated gene expression system that can be switched on with dexamethasone and switched off with tetracycline was constructed. It is based on a transcriptional activator (TGV) that consists of the *Tn*10 encoded Tet repressor, the rat glucocorticoid receptor hormone binding domain and the transcriptional activation domain of *Herpes simplex* virion protein VP16. When stably expressed in transgenic tobacco plants, it mediates dexamethasone-inducible transcription from a synthetic promoter (P_{Top10}) consisting of seven *tet* operators upstream of a TATA-box. Tetracycline interferes with induction by negatively regulating the DNA-binding activity of the TetR moiety of TGV. The boundaries of the expression window of the TGV-driven P_{Top10} reach from undetectable levels of the reporter enzyme β-glucuronidase in the absence of dexamethasone to induced levels reaching 15–20% of the Cauliflower Mosaic Virus 35S promoter ($P_{CaMV35S}$). By modifying the sequence of P_{Top10}, we generated a new target promoter (P_{Tax}) that is stably expressed over several generations and that can be activated to levels comparable to $P_{CaMV35S}$, while yielding only slightly elevated background activities.

Introduction

Regulated gene expression systems based on the features of the Tn*10* encoded Tet repressor (TetR) have been developed for a variety of organisms (Gatz, 1997) since its first use in plant protoplasts (Gatz and Quail, 1988). The favourable properties of TetR include specific binding to *tet* operator DNA and abolishment of this DNA-binding activity in the presence of low amounts of the inducer tetracycline (tc; Hillen and Berens, 1994). When expressed in transgenic plants, TetR represses a modified $P_{CaMV35S}$ encoding three *tet* operators in the vicinity of the TATA-box (Gatz *et al.*, 1992). Application of tc, either in the hydroponic system or by painting of single organs, leads to up to 500-fold induction of promoter activity. The system has been successfully used for the inducible expression of *rolB* (Röder *et al.*, 1994), *rolC* (Faiss *et al.*, 1996), *ipt* (Faiss *et al.*, 1997), a dominant negative mutant of bZIP transcription factor PG13 (Rieping *et al.*, 1994) and arginine decarboxylase (Masgrau *et al.*, 1997) in tobacco, and of S-adenosylmethionine decarboxylase (Kumar *et al.*, 1996) in potato. A major drawback of the system is that it does not function in *Arabidopsis thaliana*, which has become one of the most used model plants for molecular genetic studies. It seems that repressor concentrations required for repressing transcription cannot be tolerated in *A. thaliana*, a phenomenon that has also been reported for tomato (Corlett *et al.*, 1996) and mammalian cells (Gossen *et al.*, 1993).

As promoter-activating systems require lower levels of the regulatory protein than promoter-repressing systems (Gossen *et al.*, 1993), TetR was turned into a transcriptional activator (called either tTA or TetVP16) by fusing it to the activation domain of *Herpes simplex* virion protein 16 (VP16; Gossen and Bujard, 1992). This modification helped to circumvent TetR-related toxicity symptoms in mammalian cells and led to the establishment of a tc-dependent expression system that has a tremendous impact on elucidating gene function in mammalian systems (Shockett and Schatz, 1996). The same concept worked in transgenic tobacco plants, where low levels of tTA activate transcription from a synthetic target promoter (P_{Top10}) consisting of seven *tet* operators upstream of a TATA-box (Weinmann *et al.*, 1994). In the presence of tc, background levels are lower as compared to the tc-inducible system described above. However, the system has two disadvantages: plants have to be kept constantly on tc for turning off transcription, and the target promoter is silenced over time. Both issues have been addressed in this work.

In order to render tTA chemically inducible, we have combined it with the glucocorticoid receptor hormone binding domain (GR HBD) whilst maintaining its tc sensitivity. When GR HBD is fused to a heterologous protein, the function of the resulting fusion protein becomes regulated by steroid hormones like dexametha-

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sone (dx; Picard, 1994). It has been hypothesized that disruption of the normal functions of the wild-type protein in the absence of the hormone is due to the association of GR HBD with a complex of cellular proteins, including heat shock protein 90. Hormone binding results in the release of the cellular protein complex from the fusion protein, leading to the restoration of the normal functions of the protein. This principle has been shown to work in transgenic plants (Aoyama *et al.*, 1995; Lloyd *et al.*, 1994; Simon *et al.*, 1996). When combined with a chimeric protein consisting of the DNA-binding domain of yeast transcription factor GAL4 and the VP16 activation domain (Triezenberg *et al.*, 1988), the resulting protein GVG mediates dx-inducible gene expression in transgenic tobacco and Arabidopsis plants (Aoyama and Chua, 1997).

Here we report the successful combination of the dx-inducible with the tc-dependent expression system, resulting in a promoter that can be turned on with dx and turned off with tc. Furthermore, we describe a new target promoter (P_{Tax}) that can provide high and stable expression levels.

Results

Construction of regulatory elements

Two different transcriptional activators were constructed (Figure 1). The first construct (TGV*) contains GR HBD sandwiched between TetR and the activation domain of VP16. This construct was further modified by inserting a stretch of 28 amino acids encoding the bipartite nuclear localization sequence (NLS) of plant transcription factor TGA1b (Katagiri *et al.*, 1989; Van der Krol and Chua, 1991) between the TetR moiety and GR HBD, yielding the chimeric protein TGV. Both constructs (TGV* and TGV) were placed under the control of $P_{CaMV35S}$. The target promoter P_{Top10}, consisting of seven *tet* operators upstream of a TATA-box (Weinmann *et al.*, 1994), was used to drive expression from the intron-containing *β-glucuronidase* (*gus/int*) reporter gene (Vancanneyt *et al.*, 1990). The tail to tail orientation of both chimeric genes was chosen to increase the distance between $P_{CaMV35S}$ and P_{Top10}, thus avoiding activation of P_{Top10} by the CaMV 35S enhancer. Both constructs were transformed into tobacco (*Nicotiana tabacum*) using *A. tumefaciens* mediated gene transfer. Resulting plants were called TGV*/GUS and TGV/GUS, respectively.

Regulation of TGV-mediated gene expression in transgenic plants

In order to quickly test for dx-inducible gene expression, leaf disks of independent transformants were cultivated on shoot induction medium containing 30 μM dx. Plants yielding calli that stained blue after incubation in X-Gluc overnight were chosen for further analysis, with our main focus lying on TGV/GUS transformants, although TGV*/GUS transformants worked as well. To monitor induction at the whole plant level, four TGV transformants were adapted to hydroponic growth under greenhouse conditions and dx was added to the liquid growth medium at a final concentration of 30 μM. The hydroponic solution was exchanged every 3 days. As shown in Table 1, GUS activities of uninduced plants were indistinguishable from untransformed control plants. After 14 days of dx treatment, GUS activities accumulated up to 2000 U. This is in

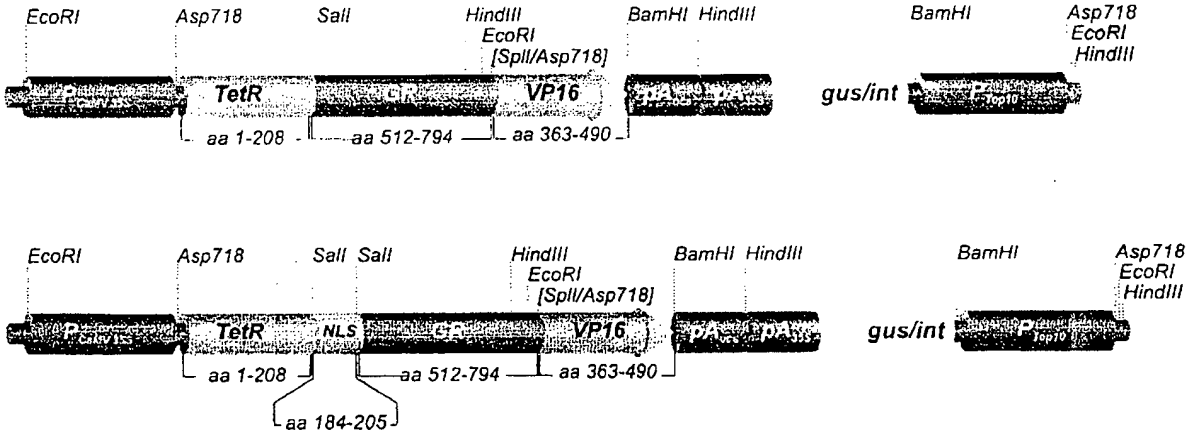

Figure 1. Diagram of chimeric genes used for the establishment of a dx-inducible/tc-inactivatable expression system.
The chimeric genes encoding the activators TGV* and TGV were placed between $P_{CaMV35S}$ and the *octopine synthase* poly-adenylation signal (pA_{ocs}). Amino acids of the different domains in the respective native proteins (TetR, GR, TGA1b, VP16) are given. The *gus'int* reporter gene was placed between the target promoter P_{Top10}, consisting of seven *tet* operators upstream of a TATA-box, and the 35S polyadenylation signal (pA_{35S}). The tail-to-tail orientation of both genes was chosen in order to avoid activation of P_{Top10} by the CaMV 35S enhancer. Restriction sites mentioned in the cloning strategy are indicated (see Experimental procedures).

Table 1. Dx-regulated expression of plants grown in hydroponic culture

Plant	−dx (0 days)	+dx (14 days)	'induction factor' (+dx/-dx)
# 21	4	2079 ± 500	x 520
# 26	5	767 ± 107	x 153
# 28	4	1842 ± 715	x 461
# 29	5	2151 ± 561	x 430
wt	7	5	-

Four independent TGV/GUS plants were grown in hydroponic culture under greenhouse conditions. A fresh solution containing 30 μM dx was supplied every 3 days for 14 days. Three leaf disks were taken from an upper, a middle and a lower leaf and analysed for GUS activity. Standard deviations refer to differences between different leaves ($n = 3$). The table indicates GUS units at time zero and at day 14 as well as 'induction factors'.

the same range as the activity of P_{Top10} when driven by tTA (Weinmann *et al.*, 1994). In average, $P_{CaMV35S}$:*gus/int* encoding transgenic plants yield approximately 10 000 U under the same assay conditions. Staining of leaves with X-Gluc revealed homogeneous induction of reporter gene expression (data not shown). In order to test for the tc sensitivity of the system, dx induction was carried out whilst painting a single leaf with tc (10 mg l^{-1}) every day using a paintbrush. After 14 days, the tc-treated leaf showed 55-fold less GUS activity as compared to the dx-induced plant, indicating that the TetR moiety of TGV still maintains its tc sensitivity in spite of the 42 kDa C-terminal extension. Painting of $P_{CaMV35S}$:*gus/int* control plants with tc did not have any effect on GUS expression levels. In summary, these experiments show that TGV confers both dx-inducible and tc-inactivatable gene expression.

Northern blots were performed to demonstrate the kinetics of the effect of the two drugs on gene expression. In 20 cm tall tobacco plants, maximal RNA levels were reached after 3 h of dx treatment (30 μM; Figure 2a). A time course of the effect of tc on gene expression is demonstrated in Figure 2(b). Two plants of the same genotype were cultivated for 14 days on dx, with the dx-containing nutrient solution being exchanged every 3 days. At time point 0, dx feeding was stopped and tc (1 mg ml^{-1}) was added to the hydroponic culture medium of one of the plants. Omission of dx did not lead to a decline of the transcript levels, indicating that after feeding of plants with dx for 14 days, the drug had accumulated to levels that led to quasi-constitutive levels of gene expression. Even after a further 6 weeks of cultivation on dx-free medium, GUS activities were still at maximal levels (data not shown). In contrast, addition of tc to the hydroponic culture led to a decline of *Gus* mRNA levels after 6 h, with no gene expression being detectable after 24 h. When using

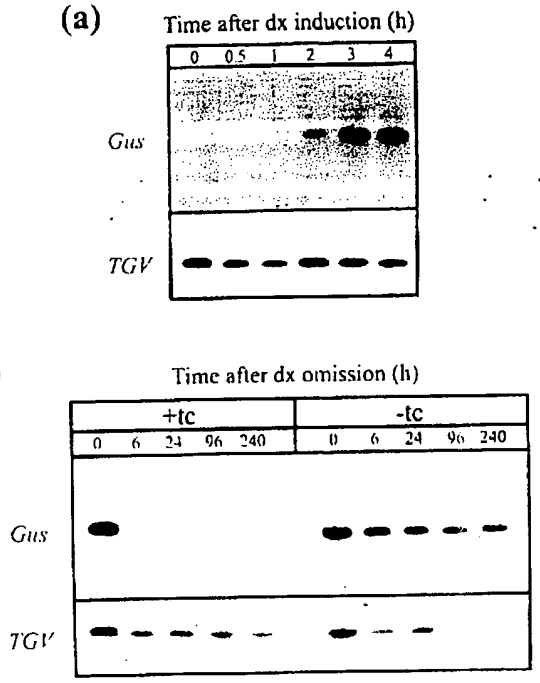

Figure 2. Kinetics of *Gus* mRNA levels regulated by either dx or tc.
(a) Kinetics of *Gus* mRNA levels induced by dx. Plant TGV/GUS#21 was adapted to hydroponic culture for 1 week. At time point 0, dx was added to the medium at a final concentration of 30 μM. Total RNA was prepared from a total of three leaf disks punched out from an upper, a middle and a lower leaf. 15 μg of RNA were loaded on each lane. Restriction fragments of the *gus* gene or the *tgv* gene were used as probes.
(b) Kinetics of the induced *Gus* mRNA after omission of dx and beginning of tc treatment. Two cuttings of plant TGV/GUS#17 carrying the *tgv* gene and the *Gus* reporter gene under the control of P_{Top10} were grown on dx for 14 days. At time point 0, the medium was exchanged against dx-free medium: tc was added and samples were collected as in (a) at the time points indicated above the lanes.

chlor-tc, the kinetics was slower, with undetectable mRNA levels being reached after 48 h (data not shown). By subsequently using dx and tc, it is now feasible to induce a pulse of gene expression for a very well-defined period of time.

Characterization of TGV expression in transgenic plants

Using Western blot analysis (Figure 3a), TGV protein levels of F_1 plants derived from four independent TGV-transformants and one highly expressing TGV* transformant were analysed. TGV levels correlated roughly with GUS activities, with the lowest expressing line TGV/GUS#26 also exhibiting lowest GUS activity (see also Table 1). Protein extracts of plants synthesising sufficient TetR or tTA to regulate the respective target promoters (Gatz *et al.*, 1991; Weinmann *et al.*, 1994) revealed that TGV* and TGV were expressed at lower levels as compared to TetR. In contrast, a signal of the expected molecular weight was missing in extracts derived from tTA transformants. As all constructs encode identical sequences between $P_{CaMV35S}$ and TetR,

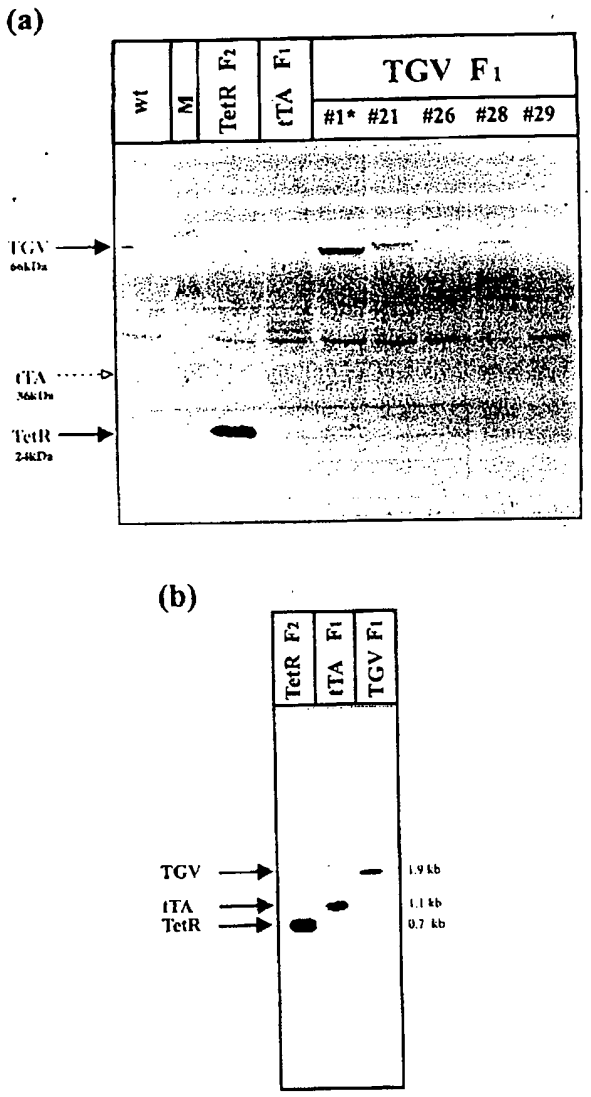

Figure 3. Expression levels of different TetR-based transcriptional regulators.

(a) Protein extracts (60 µg) of plants encoding TetR (F_2; Gatz *et al.*, 1991), tTA (F_1; Weinmann *et al.*, 1994), TGV* (F_1, highest expressing plant), and TGV (F_1; four independent plants) under the control of $P_{CaMV35S}$ were subjected to Western blot analysis using a polyclonal antiserum against TetR. The antibody detected TetR (24 kDa), TGV* (64 kDa) and TGV (66 kDa), but not tTA (36 kDa), although this plant activates transcription from target promoters P_{Tax} and P_{Top10}. Judging from a range of different Western blots, we consider the signals of MW 40–44 kDa as being unspecific.

(b) RNA was prepared from the same TetR and tTA plants as in (a) and from TGV plant #21 and analysed on a Northern blot using *TetR* as a probe. 15 µg of RNA were loaded on each lane.

we can exclude that differences in the 5′-untranslated leader might be responsible for different expression levels. RNA levels from one highly expressing line of each construct (originally selected from at least 20 independent transgenic plants) were analysed (Figure 3b). Corresponding with protein levels, TetR mRNA levels were higher than tTA and TGV mRNA levels. Although tTA was not detectable at the protein level, its mRNA was even higher than the *TGV* mRNA, indicating that tTA is less stable than TGV, at least in the absence of the effector molecules.

Toxicity of the activator and the effector molecules

Ideally, neither the activator nor the effector molecules should influence any plant function. In order to monitor any obvious toxic effects, untransformed seedlings and seedlings of TGV/GUS#21 were germinated on sterile MS medium without or with either dx, tc and chlor-tc. No difference in growth was observed. Subsequently, we continued to cultivate single plants in hydroponic culture with or without dx, tc or chlor-tc for 19 days. The phenotype of the green parts remained unaffected. However, tc and, to a lesser extent, chlor-tc caused browning of the roots, as well as reduction of root growth. In addition, we measured the efficiency of photosystem II as a very sensitive marker for possible toxic effects. Dx treatment did not have any effect on either wild-type plants or TGV transformants (F_v/F_m: 0.81 ± 0005). Tc and chlor-tc affected photosystem II efficiency by 5–15%, and 3.7–8.6%, respectively. This prompted us to test anhydro-tc, which has been shown to be an efficient inducer of TetR (Gossen and Bujard, 1993) and which has been claimed to be less toxic for plants as compared to tc (Jones *et al.*, 1998). Indeed, anhydro-tc treatment (4 mg l^{-1}) caused only 1.3–3.7% reduction of the F/F_m value. However, the apparent instability of the chemical in the hydroponic set-up makes its use rather inconvenient. We also probed the Northern blots shown in Figure 2 with the cDNA for *GNT35*, which encodes glutathione S-transferase (van der Zaal *et al.*, 1996). This gene is inducible by chemical stress, but was neither induced by dx nor tc (data not shown).

Construction and expression profile of target promoter P_{Tax}

One problem associated with P_{Top10} is the silencing phenomenon which occurred as primary transformants grew older (Weinmann *et al.*, 1994). The same effect was observed with TGV/GUS plants in this study. By avoiding potential methylation sites in P_{Tax} we aimed to construct a promoter that showed stable expression over several generations. In the 'core promoter region' between positions –48 and +1, P_{Top10} differs in 15 positions from the respective sequences of $P_{CaMV35S}$ due to the introduction of restriction sites (Weinmann *et al.*, 1994). P_{Tax} contains wild-type $P_{CaMV35S}$ sequences in this region, except at positions –36, –39 and –45. At position –45, a C was exchanged against an A in order to avoid a potential CG methylation site. The other two positions were altered to introduce a *StuI* site. In addition, the 'enhancer' portion of the promoter was redesigned, emphasis being laid on

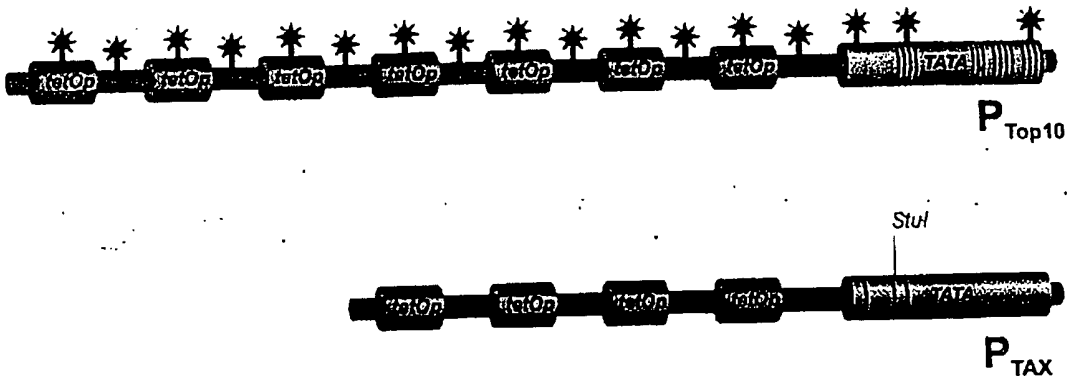

Figure 4. Schematic comparison of P_{Top10} and P_{Tax}.
P_{Top10} contains seven *tet* operators upstream of the 'core promoter' of $P_{CaMV35S}$ (–48 to +1). P_{Tax} contains four *tet* operators upstream of a 'core promoter' of $P_{CaMV35S}$ (–48 to +1). Potential methylation sites are indicated by asterisks, deviations from $P_{CaMV35S}$ (–48 to +1) are indicated by bars.

Table 2. Comparison of the expression profiles of P_{Top10} and P_{Tax}

Generation	Activator	P_{Tax}	P_{Top10}	wt	$P_{CaMV\ 35S}$
F_0	–tTA	12 (13)	7 (27)	6	9040 (5)
F_0	+tTA	10260 (19)	1340 (12)	n.d.	n.d.

The chimeric genes P_{Tax}:*gus/int*:pA_{35S} and P_{Top10}:*gus/int*:pA_{35S} were cloned into BIB and transformed into wild-type plants and plants expressing tTA. The chimeric $P_{CaMV35S}$:*gus/int*:pA_{35S} construct was introduced into wild-type plants. The number of independent transgenic plants is given in parentheses. GUS activities (pmol 4-MU min^{-1} mg^{-1} protein) were measured 6 weeks after appearance of roots in tissue culture. n.d. not determined.

Table 3. Comparison of the stability of expression of P_{Tax} and P_{Top10}

Promoter	Plant	1 year F_0	F_1	F_2
P_{Tax}	3	9252 ± 1142	16222 ± 1049	n.d.
	9	8900 ± 162	26694 ± 3330	35990 ± 2224
	13	6789 ± 355	17126 ± 1731	29472 ± 3536
P_{Top10}	5	1364 ± 331	4714 ± 432	4778 ± 667
	17	6 ± 4	186 ± 29	1232 ± 269
	18	10 ± 4	2944 ± 311	8780 ± 495

Three highly expressing plants of each construct were analysed after 1 year's cultivation in tissue culture (three independent assays from one individual plant). Three batches of 10 F_1 and 10 F_2 seedlings (2-week-old) were analysed in order to monitor GUS expression in subsequent generations.

avoiding CG or CxG sequences which can be targets for methylation-related silencing events (Ingelbrecht *et al.*, 1994). In P_{Top10}, a CxG sequence is encoded by each *tet* operator. The C of this potential methylation site encodes the central bp of the *tet* operator and does not contribute to TetR binding (Hillen and Berens, 1994). It is changed into an A in P_{Tax}. As the *tet* operator-encoding oligonucleotides in P_{Top10} were multimerized using TCGA protruding ends (Gossen and Bujard, 1992), a CG motif was found in seven positions in P_{Top10}, a feature that is also avoided in P_{Tax}. P_{Tax}, which contains four *tet* operators upstream of the –48 region of $P_{CaMV35S}$, is schematically shown in Figure 4. Its sequence is documented in Experimental procedures.

In order to compare background levels of P_{Tax} and P_{Top10} in the absence of a transcriptional activator, both promoters were transformed into tobacco (*Nicotiana tabacum* W38) using *gus/int* as a reporter gene. Analysis of 27 P_{Top10}:*gus/int* transformants confirmed that the activity of P_{Top10} is indistinguishable from the activity of untransformed control plants (Table 2; Weinmann *et al.*, 1994). Analysis of 13 P_{Tax}:*gus/int* transformants revealed a twofold higher GUS activity in these plants as compared to untransformed control plants or P_{Top10}:*gus/int* transformants.

In order to compare expression levels of both promoters in the activated state, P_{Tax}:*gus/int* and P_{Top10}:*gus/int* chimeric genes were introduced into transgenic plants expressing tTA (TGV-expressing plants were not yet available at that time point). In the presence of the activator, P_{Tax} yielded seven- to eightfold higher expression levels as compared to P_{Top10} thus reaching expression levels similar to $P_{CaMV35S}$ (Table 2). RNA analysis confirmed that the expression of P_{Tax} is reduced to levels measured in the absence of the activator when leaves are infiltrated with tc (data not shown).

As shown in Table 3, expression levels of P_{Tax} stayed constant at least through the F_2 generation. Thus, P_{Tax} provides an alternative target promoter for either tTA or TGV that yields higher and more reliable expression levels than P_{Top10}. In this analysis, P_{Top10} showed again a somewhat unreliable expression pattern, with two out of three primary transformants turning off transcription after 1 year in tissue culture. However, in the F_1 and F_2 generation this effect was reversed. The latter results

contrast with earlier results which indicated that expression levels were not rescued in the following generations. However, in those experiments activator and target genes were located on the same T-DNA, whereas in this work both constructs were introduced using two consecutive transformations.

Discussion

We have constructed a chimeric transcriptional activator that allows for the induced expression of a transgene for a very well-defined time span. Induction of the target promoter with dx leads to maximal RNA levels after 3 h in 20 cm tall tobacco plants. This response can be specifically abrogated within only 6 h after addition of tc. Alternatively, tc application to one single leaf allows for locally turning off transcription if this is required. This on/off switch is realised by combining three domains of proteins distant in evolution: the *E. coli* TetR moiety mediates tc-dependent DNA-binding to a specific target promoter, the rat GR HBD mediates dx dependence of the activation function, which is realised by the viral VP16 activation domain. The dual regulation is a feature that has not been provided by other regulated plant gene expression systems, where the off-kinetics depends both on the half-life of the drug as well as on the duration of the inducer treatment. For instance, if dx is given once to young plants for 24 h, mRNA levels decline within 2 days after omission of the drug (Aoyama and Chua, 1997). However, if plants are treated for 14 days with dx, a dx-responsive promoter stays activated for at least a further 6 weeks (this work), presumably because of the accumulation of the drug. In this situation, tc allows efficient shut down of the promoter within 6 h.

We have first characterised the potential usefulness of TGV using P_{Top10} as a target promoter. This promoter consists of seven operators localized upstream of a TATA-box and has been characterized before in combination with tTA (Weinmann *et al.*, 1994). When TGV was used in combination with P_{Top10}, no background activity was detectable using GUS activity as a reporter system, indicating that inactivation of the transcriptional activator by GR HBD is very efficient (Table 1). Induced levels reached 15–20% of $P_{CaMV35S}$, a value also reached in combination with tTA. Thus the activation potential is not diminished by the addition of GR HBD. An induction factor being defined as 'induced GUS activity' divided by 'uninduced GUS activity' minus 'GUS activity of untransformed control plants', cannot be given as the denominator is too close to zero to allow an accurate calculation. If background levels of untransformed plants are not subtracted, the 'induction factor' ranges between 150 and 520 (Table 1). X-Gluc staining of leaves and roots indicated that induction was homogeneous. When measuring GUS enzyme activity in upper, middle and lower leaves, no indication of preferential transport of dx to either sink or source leaves was obtained, at least from day 4 of dx treatment on (data not shown). However, no induction was observed in flowers, the reasons of which have not yet been elucidated. Tc abrogates induction down to undetectable *Gus* mRNA levels throughout the plant. Painting of a single leaf with tc led to a locally restricted down-regulation of the reporter gene which did not affect dx-inducible gene expression in the remainder of the plants. Down-regulation was not as stringent as in hydroponic culture, which is most probably due to less efficient tc uptake under these conditions.

P_{Top10} has been reported to be prone to silencing, at least when combined with tTA. The same problem occurred in this work using TGV. This prompted us to construct a second target promoter (P_{Tax}). In contrast to P_{Top10}, P_{Tax} contains no potential methylation sites and a –48 region that differs only in three bps from the respective region of $P_{CaMV35S}$. P_{Top10} and P_{Tax} were directly compared by transforming them into wild-type and tTA-expressing plants. At that point, TGV-expressing master plants were not yet available. In the presence of tTA, P_{Tax} yielded seven- to eightfold higher GUS activities as compared to P_{Top10}. Its strength is in the same order of magnitude as that of $P_{CaMV35S}$ (Table 2), yielding 1700-fold higher expression levels as compared to levels obtained with P_{Tax} in the absence of an activator. This background activity was twofold over background GUS activity of untransformed control plants.

The activities of P_{Tax} stayed constant for at least 1 year in the F_0 generation and remained stable in the next two generations (Table 3). P_{Top10} was silenced as primary transformants aged, but the activity was restored in subsequent generations (Table 3). These data are in contrast to data that P_{Top10} activity cannot be rescued after meiosis (Weinmann *et al.*, 1994). The difference between the two constructs is that, in this study, the activator was not localized on the same T-DNA as the target promoter. We do not know yet whether P_{Tax} would be affected if localized on the same T-DNA as tTA or TGV. Thus, we would recommend at this point to use a master plant overexpressing one of these activators for a second transformation with the target construct. As TGV mediates in combination with P_{Top10} the same expression levels as tTA, we think it likely that it does so in combination with P_{Tax}. Analysis to prove this is under way in our laboratory. We are also presently constructing new target promoters, aiming at an optimal promoter that yields background activities close to zero while maintaining high inducibility and stability of expression.

Western and Northern blot analysis revealed that the chimeric *tgv* gene was transcriptionally active in the F_1

generation (Figure 3). The relative abundance of TGV with respect to TetR was the same as in primary transformants (data not shown). In contrast, tTA was not detectable in this assay, raising the question whether tTA is less stable or more toxic than TGV. To clarify this, we performed Northern blot analysis (Figure 3b). As observed previously (Weinmann *et al.*, 1994), mRNA levels of activator plants were lower than those of TetR plants, supporting the notion of selection against highly expressing tTA and TGV transformants. *TGV* mRNA levels were lower than *tTA* mRNA levels, although TGV, but not tTA, was detectable at the protein level. This observation argues for a higher stability of TGV as compared to tTA. In addition, selection against plants expressing high *tTA* mRNA levels suggests that even low levels of tTA might be toxic, while detectable TGV levels can be tolerated by tobacco plants. This raised the question of whether TGV might elicit toxic effects after dissociation from inhibitory proteins after dx treatment.

Therefore, we performed toxicity studies with TGV-expressing plants in the presence and absence of dx. We characterised phenotype, effect on photosystem II and induction of transcription of glutathione S-transferase (*GST*) of wild-type plants and TGV-expressing plants after treatment with dx. Both processes were unaffected arguing for no severe toxicity of either TGV or dx. We also included tc treatment in this study. The phenotype of the green parts of the plants was not affected. However, tc caused a significant reduction in root growth, as described previously for tomato (Corlett *et al.*, 1996). Furthermore, photosynthetic efficiency as determined by measuring the F_v/F_m ratio of chl a fluorescence was reduced by 5–15% upon tc treatment. Chlor-tc is more favorable, causing reductions by 3.7–8.6% which may be regarded as a tolerable range for many studies under controlled growth conditions. The root phenotype was also less affected. Transcription of *GNT35*, encoding a GST that is activated by xenobiotics (van der Zaal *et al.*, 1996), was not induced by either of these treatments. In conclusion, dx treatment of TGV-expressing plants did not elicit any adverse effects, whereas tc treatment is more critical.

The construction of TGV and P_{Tax} will extend the spectrum of useful tools for the regulated expression of transgenes. We have discussed this system predominantly with regard to tc- and dx-dependent expression systems established before because of the use of the same regulatory modules. In addition, regulated expression systems responding to Cu (Mett *et al.*, 1993), ethanol (Caddick *et al.*, 1998) and the agrochemical RH 5992 (I. Jepson, personal communication) are available. The TGV-based system adds a novel tool. It has the unique property of being inducible and de-activatable by subsequent addition of the effectors dx and tc, thus allowing the regulation of transcription within a well-defined time window.

Experimental procedures

DNA constructs

Standard procedures were used for recombinant DNA analysis (Sambrook *et al.*, 1989). Plasmids derived from BIN19 (Bevan, 1984) or BIB (Becker, 1990) were used as vectors for plant transformations. The TGV* coding region was constructed by cutting pHCA/GAL4(1–93).GR.VP16 (Picard *et al.*, 1990) with *Asp*718 and *Bam*HI. The resulting 1055 bp fragment encoding GR (aa 512–794) and VP16 (aa 363–490) was cloned into pTetVP16 (Weinmann *et al.*, 1994) cut with *Spe*I and *Bam*HI to yield pTGV*. Thus, the VP16 portion of tTA was exchanged by the GR HBD-VP16 fusion of GAL4.GR.VP16. The reporter construct (P_{Top10}:*gus/int*:pA_{35S}) was excised as a *Hin*dIII fragment from pTetVP16Top10 (Weinmann *et al.*, 1994) and cloned into pTGV*, partially digested with *Hin*dIII. Plasmid pTGV*Top10 contains both genes in a tail-to-tail orientation (Figure 1). Two complementary oligonucleotides encoding the NLS (TCGAACTAGGCCTGAAAAGAAGAGAG-CTAGATTGGTTAGGAATAGGGAAAGTGCTCAACTGTCAAGGCA-AAGAAAGAAGCTCGAGT and TCGACTCGAGCTTCTTTCTTTG-CCTTGACAGTTGAGCACTTTCCCTATTCCTAACCAATCTAGCTCT-CTTCTTTTCAGGCCTAG) of TGA1b (van der Krol *et al.*, 1991) with *Sal*I overhanging ends were synthesized and cloned into pTGV*Top10 cut with *Sal*I, yielding pTGVTop10. The introduced aa acid sequence is TPEKKRARLVRNRESAQLSRQRKKLEST.

P_{Tax} was constructed by using a set of complementary oligonucleotides. First, the –48/+1 region of the CaMV 35S promoter was synthesised using oligonucleotides with *Spe*I overhanging ends at the 5′-end and *Bgl*II overhanging ends at the 3′-end. After annealing, the oligonucleotides were cloned 5′ to the *gus/int* gene (Vancanneyt *et al.*, 1990). Oligonucleotides encoding the *tet* operator sequence were synthesized with *Spe*I overhanging ends. After annealing and ligation, the reaction products were separated on a 5% polyacrylamide gel, eluted and cloned into the *Spe*I site upstream of the promoter core region. The sequence of P_{Tax}, which was constructed by using synthetic oligonucleotides, is: GAATTCGAGCTCGGTACAACTCCCACTAG-ACATATCCTACTCTATCATTGATAGAGTGACCCTTCCATAGACA-TATCCTACTCTATCATTGATAGAGTGACCCTTCCACTAGTTCTAG-ACATATCCTACTCTATCATTGATAGAGTGACCCTTCCATAGACAT-ATCCTACTCTATCATTGATAGAGTGACCCTTCCACTAGTT*CTAGT*-CTTAGCAAGGCCTTTCCTCTATATAAGGAAGTTCATTTCATTTG-GAGAA*GATC*CCCGGG. Sequences of *tet* operators and the TATA-box are underlined. The CTAG and GATC sequence marking the overhanging ends of the first pair of oligonucleotides encoding the CaMV 35S sequences are in italics. Both reporter constructs (P_{Top10}:*gus/int*:pA_{35S} and P_{Tax}:*gus/int*:pA_{35S}) were cloned into BIB which confers hygromycin resistance (Becker, 1990). The $P_{CaMV35S}$:*gus/int*:pA_{35S} construct is described by Vancanneyt *et al.* (1990).

Plant transformation, growth conditions and sampling routine

Binary vectors were introduced into *Agrobacterium tumefaciens* strain C58C1 containing pGV 2260 (Deblaere *et al.*, 1985). Leaf disks of *Nicotiana tabacum* SR1, W38 or SNN were transformed and regenerated as described by Rosahl *et al.* (1987). Plants in tissue culture were grown under a 16 h light/8 h dark regime at 28°C on Murashige and Skoog medium (Duchefa) containing 2% sucrose (2MS) and 8 g l^{-1} agar. For hydroponic growth, the nutrient medium contained 2 mM $Ca(NO_3)_2$, 2 mM $MgCl_2$, 3 mM KNO_3, 1 mM $MgSO_4$, 0.5 mM KH_2PO_4, 0.5 mM Na_2-Fe-EDTA, and

trace elements as described by Randall and Bourma (1973). Plants grown in tissue culture were adapted to hydroponic growth in the greenhouse or in the growth chamber (16 h light/8 h dark/26°C) for at least 1 week. In a typical experiment plants were 20 cm tall and did not possess more than 10 leaves. When not otherwise stated, leaf pieces of 1.2 cm in diameter were punched out from an upper, a middle and a lower leaf after chemical induction and combined for further analysis.

Application of dx and tc

Dx (Fluka) was dissolved in DMSO at 360 mM before use and diluted to 30 μM in 2MS or in hydroponic growth nutrient solution. The same volume of DMSO was added to the growth medium of control plants. Tc, chlor-tc and anhydro-tc were dissolved in water (10 m l^{-1}, 10 m l^{-1} and 1 m l^{-1}, respectively) and either added to the 2MS medium or the hydroponic buffer at a concentration of 1 mg l^{-1}, 1 mg l^{-1} and 4 mg l^{-1}, respectively. Every 3 days (dx) or every other day (tc derivatives) the hydroponic solution was exchanged. Stocks of tc derivatives should be freshly prepared each time. When painted directly on leaves, tc was used at a concentration of 10 mg l^{-1} in 0.02% Silwet L77 (Lehle Seeds) and applied every day on both sides of selected leaves using a bristle paintbrush.

Assays for GUS activity (Jefferson, 1987)

For the fluorometric GUS assay, leaf pieces of 1.2 cm in diameter were punched out, homogenized and incubated with the substrate 4-methylumbelliferyl-β-glucoronide at 37°C. Quantification of the fluorescence was undertaken using a CytoFluorII plate reader (PerSeptive). Staining of explants with 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid cyclohexylammonium) was carried out overnight at 37°C.

Western blot analysis

Total plant extracts were prepared according to Gatz *et al.* (1991) and separated on a 12.5% SDS-PAGE (Laemmli, 1970). After blotting onto PVDF membranes (Millipore) for 45 min at 140 mA, filters were washed three times for 15 min/RT with PBS-T (PBS: 58 mM Na_2HPO_4, 17 mM NaH_2PO_4, 68 mM NaCl, 0.1% v/v Tween). non-specific binding sites were saturated with 3% milk powder in PBS-T for 1 h at RT (blocking buffer). The serum directed against TetR (purified against *E. coli* extracts; Pierce) was diluted in 1:1000 in PBS-T with 0.3% milk powder and incubated with the filters for 1 h/RT. After incubation with the primary antibody, filters were washed three times for 15 min in PBS-T with 0.3% milk powder at RT. Incubation with the secondary antibody was done for 15 min with anti-rabbit Ig alkaline phosphatase conjugate (Biorad) at 1:15000 in PBS-T milk powder, 0.3%. After washing three times for 15 min at RT in PBS-T, blots were equilibrated in AP-buffer (100 mM Tris, pH 8.9, 100 mM NaCl, 5 mM $MgCl_2$). Blots were developed with 165 μg ml^{-1} 5-bromo-4-chloro-3-indolyl phosphate and 82.5 μg ml^{-1} nitroblue tetrazolium. The reaction was stopped with 0.5 mM EDTA, 20 mM Tris, pH 7.5.

Northern blot analysis

Total RNA from leaves was prepared using the Quiagen RNeasy Plant Mini Kit following the manufacturer's instructions. Blotting was done onto Nytran membranes (Schleicher and Schuell). Probes were labelled using the MultiPrime Kit from Amersham. Membranes were pre-hybridized for 30 min at 42°C in a buffer containing 50% formamide, 10% (w/v) dextran sulfate (Roth), 10% SDS, 1 M NaCl and 50 mM sodium phosphate pH 7.0. Hybridization was carried out overnight at 42°C in the same buffer after adding the probe and herring sperm DNA (0.1 mg ml^{-1}). Figures were exposed to Cronex 4 X-Ray films (Sterling).

PAM fluorometer studies

The ratio of variable to maximum chl a fluorescence (F_v/F_m) was recorded with a PAM 101 fluorometer (Walz, Effeltrich) following 10 min dark adaptation and served as a measure of photosynthetic efficiency (Krause and Weis, 1991).

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APPENDIX E

Spatial and temporal targeting of gene expression in *Drosophila* by means of a tetracycline-dependent transactivator system

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SUMMARY

In order to evaluate the efficiency of the tetracycline-regulated gene expression system in *Drosophila*, we have generated transgenic lines expressing a tetracycline-controlled transactivator protein (*tTA*), with specific expression patterns during embryonic and larval development. These lines were used to direct expression of a *tTA*-responsive promoter fused to the coding region of either the β-galactosidase or the homeotic protein Antennapedia (ANTP), under various conditions of tetracycline treatment. We found that expression of β-galactosidase can be efficiently inhibited in embryos and larvae with tetracycline provided in the food, and that a simple removal of the larvae from tetracycline exposure results in the induction of the enzyme in a time- and concentration-dependent manner. Similar treatments can be used to prevent the lethality associated with the ectopic expression of ANTP in embryos and, subsequently, to control the timing of expression of the homeoprotein ANTP specifically in the antennal imaginal disc.

Our results show that the expression of a gene placed under the control of a tetracycline-responsive promoter can be tightly controlled, both spatially by the regulatory sequences driving the expression of *tTA* and temporally by tetracycline. This provides the basis of a versatile binary system for controlling gene expression in *Drosophila*, with an additional level of regulation as compared to the general method using the yeast transcription factor GAL4.

Key words: Tetracycline, Gene expression, *Drosophila*, *Antennapedia*

INTRODUCTION

An essential and general experimental approach to analyse the function of a gene in a whole organism is to examine the phenotypic consequences of its directed expression in certain cells, or at a developmental stage, in which the gene is normally silent. In *Drosophila*, two major systems have been designed to achieve the conditional expression of gene constructs integrated into the genome. In the first, the coding sequence of the gene of interest is placed under the control of promoters inducible according to the culture conditions. The *hsp70* gene promoter is commonly use for that purpose (see Schneuwly et al., 1987 and references therein). High levels of induction can be obtained at well-defined time periods during development upon exposure of the organism to elevated temperatures. This advantage is often limited because ectopic expression occurs in all cells and endogenous genes are repressed during heat shocks. Consequently, side defects including lethality may mask the result of the ectopic expression in the desired cell type(s). It is also frequently necessary to repeat heat shocks over an extended time period to observe the phenotypic consequences, and it may be difficult to distinguish between the primary and secondary effects of the overexpression (e.g. Gibson and Gehring, 1988).

An alternative approach is to engineer a gene construct inducible by a single transcription factor whose activity can be controlled *in vivo*. The ability of the yeast transcription factor GAL4 to activate transcription in *Drosophila* (Fisher et al., 1988) has been exploited to generate a versatile method for targeting gene expression in this organism (Brand and Perrimon, 1993). Any gene of interest can be placed under the control of a promoter containing GAL4 upstream activating sequences (UAS) and integrated stably into the genome of a parental line (responder strain), since it remains silent in the absence of GAL4. Tissue-specific expression of the gene is obtained upon crossing to a second parental line (driver strain) expressing the transcription factor under the control of a suitable promoter. Although a large number of strains expressing GAL4 in a wide variety of patterns can be selected on the basis of the expression of a reporter gene bearing the UAS sequences (Brand and Perrimon, 1993; Yeh et al., 1995; Calleja et al., 1996), only a few of them have been used to direct expression of functional proteins in the post-embryonic stages of development (Brand and Perrimon, 1993; Capdevila and Guerrero, 1994; Hinz et al., 1994; Speicher et al., 1994; Rimmington et al., 1994; Ferver et al., 1995; Halder et al., 1995; Zink and Paro, 1995; Freeman, 1996; Morimura et al., 1996). The limit in this binary system lies in the lack of

temporal control, which remains primarily determined by the regulatory sequences driving GAL4. Because most of the gene-specific enhancers are active at various stages of development in *Drosophila*, GAL4-mediated induction is frequently observed from the embryonic stage onwards, and often results in premature lethality (e.g. Halder et al., 1995). To overcome this difficulty, Flp-mediated recombination has been used to achieve conditional expression of a transgene upon recombination of an FRT cassette separating the coding region from a promoter (Struhl and Basler, 1993). This approach can be used to control the expression of GAL4 (Pignoni and Zipursky, 1997). In any case it requires the combination of various specific constructs and it can be applied only to tissues in which cell division occurs, since it results in the generation of clones of expressing cells.

Extension of the more versatile binary system for post-embryonic studies could be achieved with the use of a regulatory protein modulated in the organism with an innocuous effector. One of the best candidates is the tetracycline-dependent transactivator (*tTA*) comprising the tetracycline repressor of *E. coli* (*tetR*) and the strong transcriptional activation domain of the herpes simplex virus protein VP16 (Gossen and Bujard, 1992). The high affinity and specific binding of *tetR* to the tetracycline operator sequences (*tetO*) can be inhibited by tetracycline (Hillen and Wissmann, 1989) and is thought to result from a conformational change of *tetR* upon association with tetracycline (Hinrichs et al., 1994). In HeLa cells, *tTA* was found to stimulate transcription of a promoter bearing a multimerized *tetO* by several orders of magnitude, and a fast and reversible switch of the *tTA*-dependent promoter was obtained upon addition or removal of tetracycline from the culture medium (Gossen and Bujard, 1992). These features have been extensively exploited in tissue culture where tetracycline levels can be tightly controlled. Tetracycline-regulated expression of reporter genes was also demonstrated in whole plants (Weinmann et al., 1994) and in transgenic mice (Hennighausen et al., 1995; Kistner et al., 1996). In the latter case, the efficiency of tetracycline, administrated by slow-release tetracycline pellets or in drinking water, has been mostly evaluated by analysing the expression of sensitive reporter genes, although a few examples of successful expression of proteins have been reported (see Shockett and Schatz, 1996 for a review).

In this study we determined the functional properties of *tTA* in *Drosophila* by expressing this regulatory protein under the control of various promoters. Using a *lacZ* reporter gene placed under the control of a promoter bearing seven copies of *tetO*, we found that expression of β-galactosidase can be tightly controlled in embryos and larval tissues. Furthermore, we used *tTA*-expressing strains and tetracycline treatments to drive tissue-specific ectopic expression of the homeoprotein *Antennapedia (Antp)* at different stages of development. Our results demonstrate the usefulness of the *tet* system in *Drosophila*.

MATERIALS AND METHODS

DNA constructs

Most of the constructs were assembled by multiple step cloning according to standard methods (Sambrook et al., 1989). Further details and maps are available upon request.

tTA driver constructs

hsp70-tTA

The *tTA* coding region was isolated as a 1.1 kb *Eco*RI-*Bam*HI fragment from pUHD 15-1 (Gossen and Bujard, 1992) and cloned into CaSpeR-hs (Thummel and Pirrotta, 1992).

RHT (*rosy*, *hsp70* promoter, *tTA*)

This P element vector derives from the enhancer-test vector HZ50PL (Hiromi et al., 1985). The *tTA* coding region is flanked by the minimal promoter and the poly(A) sequences of *hsp70* (Fig. 1A).

ey-tTA and *HoxA7-tTA*

The *eyeless (ey)* gene enhancer (a 3.5 kb *Kpn*I fragment from *ey* Eco 3.6) (B. Hanck, T. Eggert, W. J. Gehring and U. Walldorf, unpublished) and a 630 bp fragment of the intron of the *HoxA7* gene from pB6 (Haerry and Gehring, 1996) were cloned in RHT to give *ey-tTA* and *HoxA7-tTA*, respectively.

Tetracycline-responder constructs

tetO-lacZ

The heptameric repeat of the tet operator was isolated as a 310 bp *Eco*RI-*Kpn*I fragment from pUHC 13-3 (Gossen and Bujard, 1992) and cloned upstream of the P-*lacZ* fusion of the enhancer-test vector CPLZ (Wharton and Crews, 1993). CPLZ contains the P-element transposase promoter (up to −42 from the cap site) and the N-terminal transposase sequence fused in-frame with *lacZ* and the polyadenylation signal of *hsp70*.

WTP (white-*tetO*-P promoter)

This P-element vector was constructed to express any gene under the control of a tetracycline-responsive promoter. It contains the vector backbone of CPLZ, the heptameric repeat of the tet operator, the P-element promoter and leader sequences from Carnegie 4 (Rubin and Spradling, 1983) and the polyadenylation signal of SV40.

tetO-Antp and *tetO-AntpΔHD*

The cDNAs encoding a full-length ANTP protein or a variant with a deletion of the homeodomain were isolated as *Not*I fragments from pHSSΔA and pNHT-A11, respectively (Gibson et al., 1990) and cloned into the corresponding site of WTP.

Germline transformation and *Drosophila* strains

P-element mediated transformation of ry^{506} or *y ac* w^{1118} recipient strains was carried out essentially as described (Spradling, 1986). A description of the markers and balancer chromosomes indicated in Figs 3 and 4 can be found in Lindsley and Zimm (1996). A405.1 M2 and rK781 have been described (Wagner-Bernholz et al., 1991; Flister, 1991).

Tetracycline media and treatments

A tetracycline-containing medium suitable for larval feeding and maintenance of adults was obtained by mixing 100 ml of tetracycline solution (tetracycline hydrochloride (Sigma) diluted with sterile water at the required concentration), 25 g of Instant *Drosophila* Food (Carolina Biological Supply) and 1 g of dry yeast.

Tetracycline was provided to adult females by placing 50-70 virgins in a glass vial containing a 2.5 cm filter (Whatman grade 3 MM) soaked with 500 μl of a 4% sucrose solution with tetracycline at the appropriate dilution. Females were fed for 3-4 days, following a daily cycle of 16 hours on tetracycline-containing filters and 4 hours on standard food supplemented with a drop of yeast paste. Males of the relevant genotype were placed with females on tetracycline and allowed to mate overnight before the beginning of egg collections. For

experiments requiring larval feeding, batches of 100-200 eggs harvested from grape juice plates were placed on pieces of nylon mesh, and allowed to develop at 25°C on tetracycline-containing food. When necessary, larvae were separated from their food by floating in 30% glycerol, collected with forceps, washed with PBS and transferred on standard food in groups of 50 to 100.

Phenotypic analyses

Embryos and larval tissues were fixed and stained for β-galactosidase as described (Bellen et al., 1989). Adult heads were separated from the body of narcotised flies and holes were made into the cuticle to facilitate penetration of the fixative. For antibody staining of embryos and examination of cuticular phenotypes, standard procedures were applied (Ashburner, 1989).

RESULTS

We have designed a general system to express *tTA* under the control of regulatory sequences (RHT driver construct), in order to direct the expression of a gene of interest under the regulation of a tetracycline-responsive promoter (WTP responder construct). The gene constructs can be stably propagated into the genome of separate strains, and *tTA*-dependent gene induction is obtained in the F_1 offspring of the cross where it can be controlled by tetracycline (Fig. 1A).

tTA is a potent transactivator in *Drosophila*

In order to analyse the transactivation potential of *tTA*, we have used an indirect heat shock assay to drive ubiquitous expression of *Antp* in embryos. Heat shock assays were performed on embryos carrying *tTA* under the control of the *hsp70* gene promoter (*hsp70-tTA*) and a WTP derivative carrying a full-length *Antp* cDNA (*tetO-Antp*). Independent transformants were found to give an identical embryonic phenotype to the H4 line that carries a direct *hsp70-Antp* construct (Fig. 1B; see also Gibson and Gehring, 1988 for a complete description of the H4 line). In contrast, heat-shocked embryos carrying a WTP derivative with a deletion of the homeodomain (*tetO-Antp*ΔHD) or the empty WTP vector (*tetO−*) showed a wild-type cuticle and developed to the adult stage (Fig. 1B and data not shown). A western blot of embryonic extracts prepared from heat-shocked embryos and probed with an ANTP-specific monoclonal antibody (Condie et al., 1991) reveals similar levels of ANTP expression (Fig. 1C). In addition, transformation of the adult antenna into a mesothoracic leg can be obtained when heat shocks are applied to third instar larvae (Gibson and Gehring, 1988; D. Resendez-Perez, B. Bello and W. J. Gehring, unpublished). Taken together, these results show that *tTA* can activate transcription of a promoter that contains *tetO* sequences without any toxic effect of this regulatory protein in *Drosophila*.

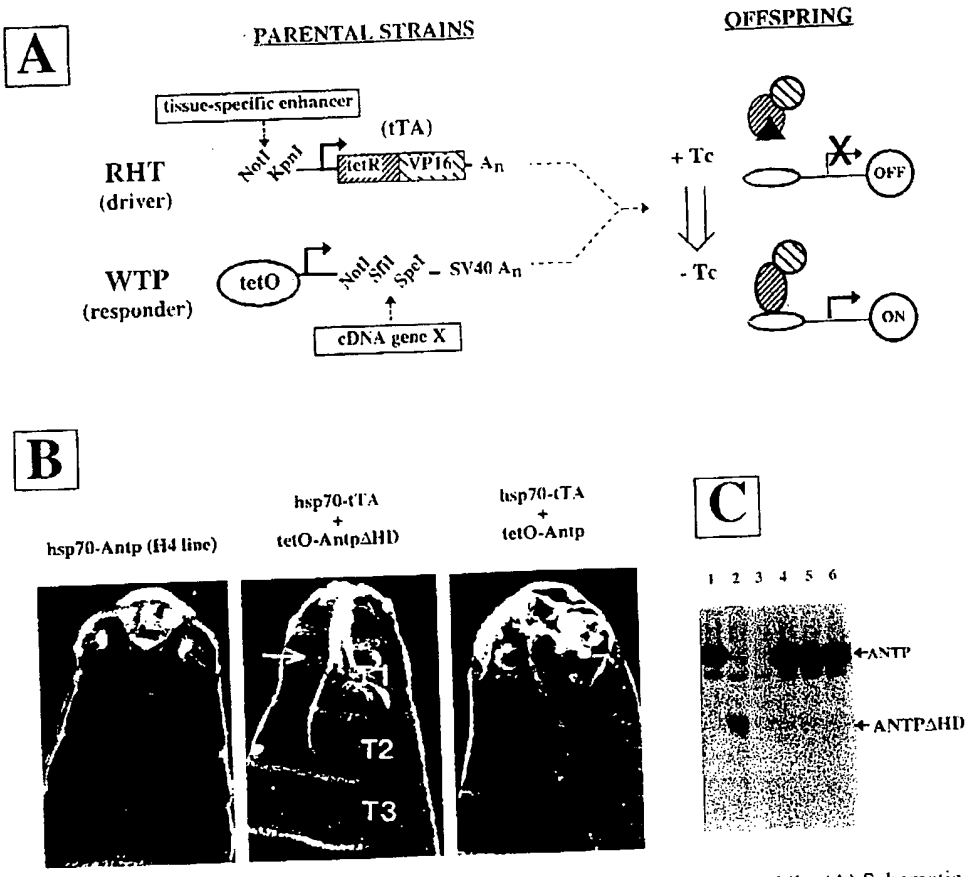

Fig. 1. Transgenic constructs and the transactivation potential of *tTA* in *Drosophila*. (A) Schematic representation of the tetracycline-inducible system in *Drosophila*. The transformation vectors RHT and WTP are, respectively, designed to express *tTA* under the control of enhancer-like elements and any cDNA under the regulation of a *tTA*-responsive promoter that contains *tetO* sequences. Derived constructs are integrated and propagated in two separate parental strains. In absence of tetracycline (−Tc), the binding of *tTA* results in the activation of gene X with the spatial and temporal specificities determined by the driver construct (on state). Raising the offspring on a tetracycline-containing medium is used to prevent the binding of *tTA* in order to keep the responder construct silent up to a certain point of time (off state). Stopping tetracycline exposure allows time-specific induction of gene X upon withdrawal of the antibiotic. (B) Cuticular preparations of embryos heat shocked for 30 minutes at 6.5 hours of embryogenesis. All embryos carry a single copy of the constructs indicated at the top. Note the similar defect in head evolution in *hsp70-Antp* and *hsp70-tTA*; *tetO-Antp*, and the T1 to T2 transformation when compared to the wild-type cuticle of *hsp70-tTA*; *tetO-Antp*ΔHD embryos. The identical segmental transformation is revealed by the disappearance of the characteristic denticle belt of the first thoracic segment (white arrow), indicating a transformation of T1 towards T2. The head segments are also transformed towards T2. T1, T2, T3: first, second and third thoracic segments respectively. (C) Detection of the ANTP proteins by western blot analysis in crude extracts of embryos heat shocked for 2 hours with a 4 hour recovery. Lane 1, *hsp70-Antp* embryos (H4 line); lanes 2- 6, embryos carrying *hsp70-tTA* and either *tetO-Antp* ΔHD (lane 2) or the empty WTP vector (lane 3), or independent insertions of *tetO-Antp* (lanes 4-6).

Tetracycline-controlled expression of *lacZ* during larval development

In order to express *tTA* during larval development, we inserted the eye-specific enhancer of the *ey* gene (Quiring et al., 1994) into the vector RHT and generated *ey-tTA* transformants. The expression of *tTA* was detected specifically in the eye imaginal disc by means of a *lacZ* reporter gene placed under the control of a promoter bearing *tetO* sequences (Fig. 2). Detection of β-galactosidase activity in the eye disc of the larvae reveals two essential features. First, enzymatic activity is detectable within less than 15 minutes of incubation with X-gal revealing a high level of expression of *tTA*. Second, this activity is detected in the eye disc over an extended period of development with a dynamic pattern (Fig. 2A, top row). In the early third instar, expression is detected uniformly in the eye part of the eye-antennal disc (left panel), which corresponds to the undifferentiated cells of the eye epithelium (Ready et al., 1976). The same pattern was observed in second instar larvae (not shown). Eye discs stained at different times during the third instar show that β-galactosidase activity gradually fades in the anterior region of the disc as the morphogenetic furrow moves anteriorly.

To determine whether tetracycline incorporated in the larval food could inactivate *tTA*, we stained early third instar larvae raised on media containing increasing concentrations of the antibiotic (Fig. 2A, time 0). No activity could be detected in the eye-disc of larvae exposed to as little as 0.1 μg/ml even after prolonged staining (24 hours) in X-gal solution (Fig. 2A, left column). Lower concentrations failed to inactivate lacZ expression (not shown). The results indicate an efficient uptake of tetracycline from the food and its diffusion to the imaginal discs, leading to a complete inactivation of *tTA* in a concentration-dependent manner. The same dose-response was obtained for larvae exposed to tetracycline for approximately 2 more days, suggesting that the concentration of tetracycline in the larval haemolymph does not change dramatically during the feeding period (not shown). In contrast, stopping the larval exposure to tetracycline was expected to decrease the level in the haemolymph and to restore the ability of *tTA* to bind and transactivate the *lacZ* reporter gene. To test this hypothesis, early third instar larvae were transferred to standard food, fixed and stained every 6 hours for β-gal activity (Fig. 2A). Enzymatic activity was detected 6 hours after the removal of the larvae from the medium containing 0.1 μg/ml tetracycline (Fig. 2A, second row from the top) or in a range of 18 to 24 hours when the larvae were exposed to 1 μg/ml or 10 μg/ml tetracycline, respectively (Fig. 2A, third and fourth row from the top). After induction, 12-24 hours were necessary to obtain

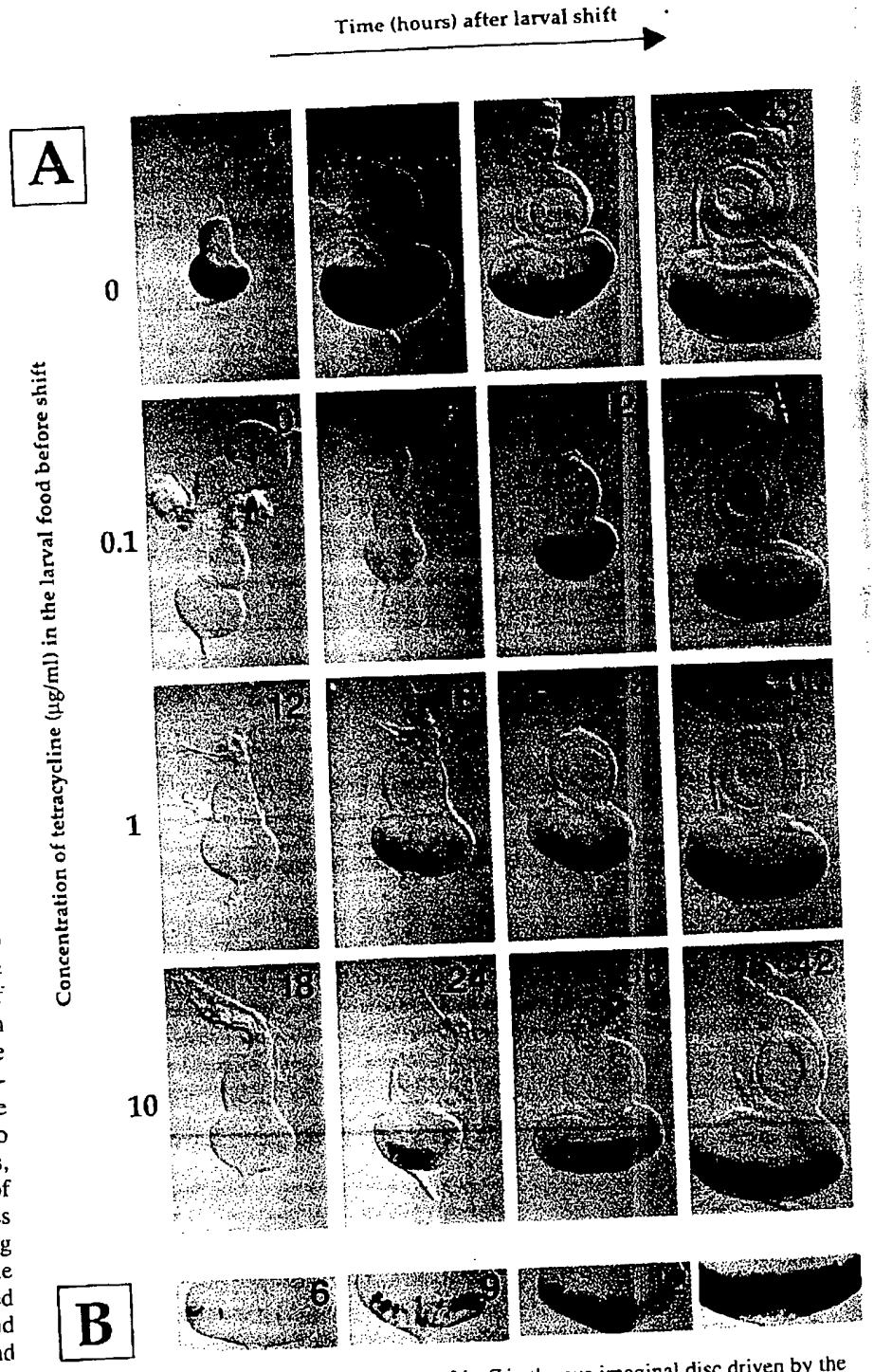

Fig. 2. Tetracycline controlled expression of *lacZ* in the eye imaginal disc driven by the *ey-tTA* strain. (A) Larvae of the genotype *ey-tTA/+; tetO-lacZ/+* were raised in the presence of increasing concentration of tetracycline, shifted to standard food at the early third instar and dissected at different times after shifting, indicated in hours at the top right of every figure. (B) Temporal profile of *lacZ* induction from larvae removed from 0.1 μg/ml tetracycline during the late third instar. Note the increasing number of cells expressing *lacZ*.

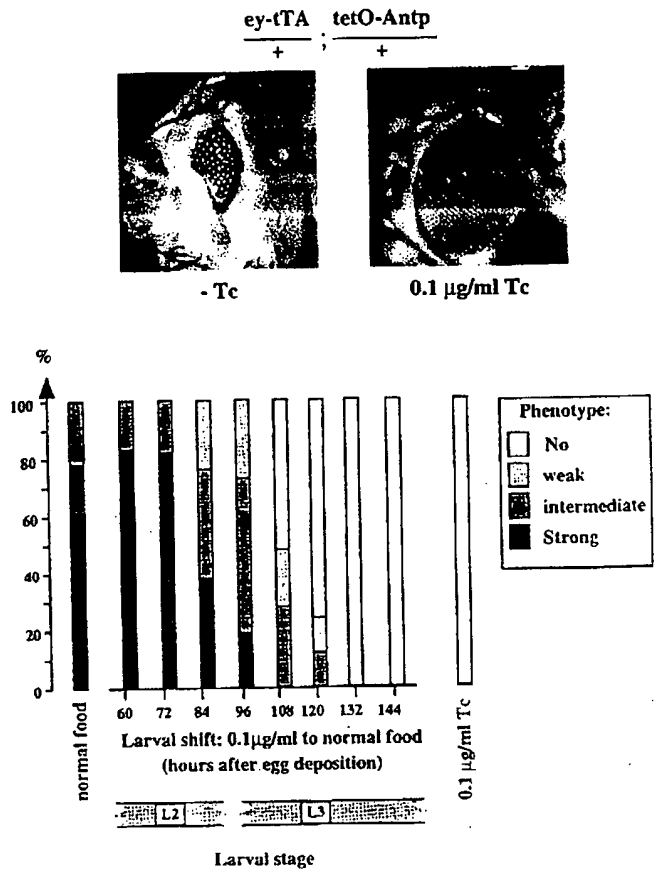

Fig. 3. Induction of *Antennapedia* in the eye imaginal disc by means of tetracycline. (Top) Eyes of adult flies raised in absence (−Tc) or in the presence of 0.1 µg/ml tetracycline (Tc) in the larval food. (Bottom) Semi-quantification of the eye phenotype in adults derived from larvae raised with 0.1 µg/ml tetracycline and shifted to standard food at 12 hour intervals. At least 50 adults were scored at every time point. Larval stages were identified on the basis of the morphology of the tracheal system and the anterior spiracles.

a comparable staining intensity in imaginal discs isolated from larvae exposed to tetracycline and control larvae. These data show that the withdrawal of the larvae from tetracycline is followed by a rapid induction of the *tetO-lacZ* transgene under the control of *tTA*. The concentration- and time-dependent expression of *lacZ* is likely to reflect the need to lower the concentration of tetracycline in the disc cells below a certain threshold level, which allows *tTA* to bind the tet operator and to stimulate transcription of the promoter. A careful examination of the staining patterns also suggested that the *tetO-lacZ* transgene was not turned on in every cell at the same time after withdrawal of tetracycline (Fig. 2A). To confirm this observation, we performed larval shifts during the second half of the third instar, when expression of *tTA* is uniform in the posterior part of the eye disc. Upon removal of the larvae from 0.1 µg/ml tetracycline, induction of *lacZ* can clearly be observed in a gradually increasing number of cells (Fig. 2B). Similar observations were obtained with different *tTA*-expressing strains, suggesting variations in the kinetics of the clearance of the antibiotic and/or the trancriptional activation.

Tetracycline-controlled expression of *Antennapedia* targeted to the eye disc

To determine the relevance of the data obtained with the *lacZ* reporter gene we used the same driver strain to direct expression of *Antp* under various conditions of tetracycline treatment. In the absence of tetracycline, adults obtained from

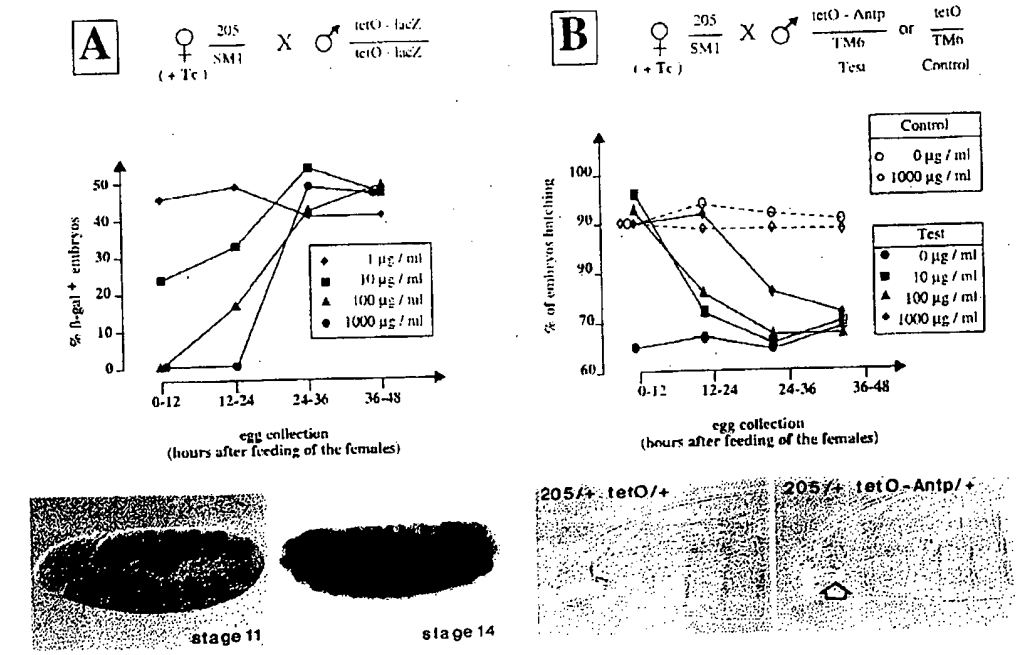

Fig. 4. Inhibition of *tTA*-dependent gene expression in embryos by maternal transmission of tetracycline. (A) Repression of the *lacZ* reporter driven by the 205 strain, shown at the bottom by immunodetection of β-galactosidase. Embryos collected over successive 12 hour periods following the feeding of 205/SM1 females with tetracycline, were stained for β-gal activity. At least 100 embryos older than the germ band extended stage (stage 11) were scored at every time point. 50% of transheterozygotes were expected in the offspring of the cross indicated at the top. (B) Tetracycline-dependent rescue of embryonic lethality by repression of the *tetO-Antp* transgene. Embryos collected over 12 hour periods after feeding of their mothers with tetracycline were allowed to develop at 25°C and the percentage of hatching larvae determined. At least 200 embryos were scored at every time point. 25% of transheterozygotes are expected from the cross indicated at the top. *tetO* refers to an insertion of the empty vector WTP on the third chromosome, used as a control. Bottom: cuticular phenotype of embryos of the indicated genotype. Note the failure of head involution marked by the open arrow in embryos expressing *Antp* under the control of the 205 strain.

a cross between the parental *ey-tTA* and *tetO-Antp* strains showed reduced and irregular compound eyes (Fig. 3, –Tc). Since *Antp* is normally not expressed in the eye disc (Wirz et al., 1986), its expression in this tissue would interfere by unknown mechanisms with the normal development of the eye. No other morphological defects could be detected in adults, in agreement with the eye-specific expression of *tTA* detected with the *lacZ* reporter. To ascertain that the eye phenotype resulted from the ectopic expression of *Antp* in the eye disc, we raised larvae on a medium containing tetracycline at various concentrations. The eyes were restored to a wild-type appearance with 0.1 μg/ml tetracycline (Fig. 3, top) but not with 0.01 μg/ml tetracycline, in good agreement with the dose-dependent repression of the *lacZ* reporter (Fig. 2A).

As indicated with the *lacZ* reporter, the directed expression of *Antp* by the *ey-tTA* strain should occur continuously throughout larval development and shift rapidly during the third instar when the cells undergo differentiation. We used tetracycline to control the timing of *Antp* overexpression in order to determine the functional significance of this dynamic pattern of expression, with respect to the alteration of the eye development. Newly hatched larvae were first fed with 0.1 μg/ml to inhibit *tTA* activity and then transferred to a standard medium every 12 hours to induce *Antp*. Adults were scored for eye defects and classified according to an arbitrary scale of strong, intermediate, weak or no detectable eye phenotype (Fig. 3, bottom). All the adults derived from larvae exposed to tetracycline up to the second instar showed strong eye defects in a range indistinguishable from their siblings raised in absence of tetracycline. In contrast, subsequent shifts allowing induction of *Antp* from the early third instar onward led rapidly to a complete rescue of the eye morphology. These results confirm the efficiency of the tetracycline control with a functional homeoprotein and suggest that the alteration in the normal eye development is mostly dependent on the ectopic expression of *Antp* in the undifferentiated cells of the eye epithelium.

Repression of *tTA*-dependent gene expression in embryos by maternal transmission of tetracycline

The embryonic development of *Drosophila* is not easily amenable to antibiotic treatment since the egg is protected by an impermeable set of eggshells but it is relatively fast (22-24 hours at 25°C) and maternal components are transmitted to the oocyte by the nurse cells and the follicle cells in the female ovaries. The influence of tetracycline given to the parental females was first tested on the strong *lacZ* expression driven by the *tTA* construct of the line 205 (Fig. 4A). This driver line 205 was isolated among twelve independent transformants of the *HoxA7-tTA* construct (see Materials and Methods) because of its unique expression pattern observed in the antennal disc (Fig. 5), the leg discs, the central nervous system, the epidermis and various internal tissues (not shown). Since the other lines showed a reproducible pattern in the eye disc and the larval brain due to the *HoxA7* enhancer (not shown), the line 205 is likely to reflect a modified expression of the transgene under the influence of genomic regulatory sequences flanking the integration site (Wilson et al., 1990). When assayed in embryos with the *lacZ* reporter gene, expression of *tTA* in the 205 strain starts at the end of germ band extension, about 5 hours after egg laying (AEL), and is detected mostly in the trunk region with a segmentally repeated pattern (Fig. 4A, bottom). This pattern changes rapidly, so that at the end of germ band retraction (approximately 10 hours AEL), strong expression is detected all over the ectoderm. The staining appears patchy in the cephalic segments and is not uniformly distributed in the thoracic and abdominal segments (Fig. 4A, bottom). After treatment of the females with tetracycline (see Materials and Methods), repression of the *lacZ* reporter is mostly effective in the eggs collected immediately after the end of exposure to the antibiotic and is dose-dependent (Fig. 4A, top). Repression was obtained in 100% of the eggs collected within 12 hours after the treatment of the females with 100 μg/ml or more of the antibiotic, and in a large fraction of them with 10 μg/ml. The gradual loss of repression observed in the eggs collected later is likely to reflect a decrease of the maternal pool of tetracycline accompanying the continuous production of eggs.

The same procedure of tetracycline treatments was tested for its effect on the survival rate of embryos carrying the driver construct 205 and either a *tetO-Antp*, or an empty responder construct (*tetO–*), as control (Fig. 4B). Examination of the embryonic cuticles revealed major defects in the formation of the head (Fig. 4B, bottom), a phenotype reminiscent of heat-shocked embryos in which *Antp* was ubiquitously expressed (Fig. 1B), although no homeotic segmental transformations were observed. This might reflect a different level of induction of *Antp* as compared to the use of a heat shock promoter but it is more likely to be due to a difference in the timing and the spatial expression of the homeoprotein, since transformations obtained by heat shocks are optimal when induced at 5-7 hours of development (Fig. 1B, see also Gibson and Gehring, 1988), at a stage when the 205 driver is mostly active in the trunk and is not ubiquitously expressed (Fig. 4A, bottom). Nevertheless, line 205 allowed us to test the effect of tetracycline on the survival rate of the embryos and, as shown in Fig. 4B, the embryonic lethality could be overcome in a dose-dependent manner by providing tetracycline to the females. The embryonic rescue was in good agreement with the tetracycline-mediated repression of the *tetO-lacZ* transgene (Fig. 4). These two independent assays clearly demonstrate the possibility to inactivate *tTA* in embryos in order to keep a promoter silent during this stage of development.

Targeted mis-expression of *Antp* in larvae following embryonic rescue

The efficient inactivation of *tTA* in embryos prompted us to analyse the fate of tetracycline-rescued embryos in more detail. As expected, embryos did not develop to the adult stage in the absence of tetracycline in the larval food, whereas addition of tetracycline led to the recovery of viable adults in a concentration-dependent manner (the quantitative data are available upon request). In addition, larvae raised under optimal conditions were shifted to standard food at various times to allow induction of *Antp*. Shifts performed during the late third instar led to the recovery of pharates or adults, whereas larvae shifted earlier essentially failed to undergo metamorphosis. Examination of the adults revealed very specific morphological modifications of the antennae and the head vertex (Fig. 5K-M), in the area expressing the *lacZ* reporter under the regulation of the 205 driver line (Fig. 5E-G). In contrast, transheterozygotes raised continuously with 10 μg/ml tetracycline showed wild-type structures (Fig. 5H-J),

demonstrating the highly specific alterations in the development of adult flies following the mis-directed expression of *Antp* by the 205 line. As revealed with the *lacZ* reporter, expression of *tTA* follows a dynamic spatial pattern in the primordia of the antenna from the mid-third instar onwards (Fig. 5A-D) and in the presumptive area of the occelli from the larval/pupal transition onward (Fig. 5C,D). Moreover, the lag in induction imposed by the removal of the larvae from tetracycline exposure suggests that the alterations of adult structures mostly result from the ectopic expression of *Antp* during the pupal stage. No defects were found in the legs or the palps where the 205 driver is also strongly expressed (not shown), in agreement with previous observations showing that only the derivatives of the eye-antennal disc respond to the ubiquitous expression of *Antp* induced by heat shock (Gibson and Gehring, 1988). These latter studies showed that repeated pulses of heat-shock expression are required during the third larval instar to achieve complete antenna to leg transformations. Our results confirm previous observations showing that the late larval induction of *Antp* does not induce fully differentiated morphological markers of the leg (Scanga et al., 1995; Larsen et al., 1996). Although we observe different arrangements of bristles on the antenna, none of them showed the bracts characteristic for leg bristles.

Directed expression of *Antp* in the antennal disc by *tTA* activates *rK781*

Since the observation of adult phenotype required late larval shifts, we asked whether the consequences of *tTA*-dependent expression of *Antp* could be directly assayed in the imaginal discs. As a marker, we used the enhancer detector line rK781, which was isolated in a screen for *Antp*-regulated genes on the basis of their response to the overexpression of the ANTP homeoprotein in the eye-antennal disc (Wagner-Bernholz et al., 1991). We combined driver, responder and test constructs in larvae, exposed them to tetracycline treatment, and assayed β-galactosidase expression in wandering third instar larvae. When raised continuously with 10 μg/ml tetracycline, the normal pattern of rK781 expression was detected in all the discs (not shown) and in a few cells of the antennal disc (Fig. 6, left), as previously described (Wagner-Bernholz et al., 1991; Flister, 1991). When dissected from larvae that were removed from tetracycline exposure, *lacZ* expression could be reproducibly detected in the form of a crescent at the border between the arista and the third antennal segment (Fig. 6, middle). This area corresponds to the most proximal part of the wedge-shaped sector expressing *tTA*, as visualised with the *lacZ* reporter (Fig. 6, right) and is also the first to express *tTA* during third instar (Fig. 5B). These results show that derepression of *Antp* by removal of tetracycline can be demonstrated by the activation of a downstream target gene in the antennal disc. These findings indicate that the tetracycline-dependent expression system efficiently repressed *Antp* in embryos and allows subsequent derepression in imaginal discs.

DISCUSSION

In this study, we report a detailed evaluation of the different properties of the tetracycline-dependent gene expression system in *Drosophila*. Since its description in transformed HeLa cells (Gossen and Bujard, 1992) this regulatory system has been extensively used in cell culture. In higher eucaryotes including plants and mouse, tetracycline-controlled activity of *tTA* has been mostly evaluated on reporter genes (see Shockett and Schatz, 1996 for a review), although a few examples of successful expression of proteins have been reported (Efrat et al., 1995; Ewald et al., 1996; Mayford et al., 1996; Shockett et al., 1995; St-Onge et al., 1996). By using *Drosophila* lines expressing high levels of *tTA*, we show that the strong induction of the *lacZ* reporter can be efficiently prevented by tetracycline in both embryos and imaginal discs. We have evaluated the dose-response and defined easy and reliable protocols of tetracycline treatment to control the repression of the *lacZ* reporter gene. Furthermore, we also show that this system is fully functional to control the spatial and temporal expression of the ANTP homeoprotein. The lines *ey-tTA* and 205 described in this study show the highest levels of *tTA* among the lines generated to date in the laboratory and we have reproducibly obtained repression of gene activity in embryos by feeding their mothers with tetracycline in a range from 1 to 1000 μg/ml tetracycline, and in larvae, with as little as 0.1 μg/ml tetracycline. The use of tetracycline is especially appropriate to keep the inducible gene promoter silent during embryogenesis in order to direct its expression during larval development. Tetracycline concentrations ranging from 0.1 to 10 μg/ml ensure reactivation of the tetracycline-responsive promoter within 24 hours after transfer of the larvae to normal medium. It is important to point out that the amount of tetracycline required to inactivate *tTA* is both low and non-toxic. This is essential to keep the promoter inactive up to a desired stage and to ensure its fast activation upon removal from tetracycline exposure. We have found that the addition of tetracycline to the larval food does not give any toxic effect in a range of 0 to 100 μg/ml, although the development is slowed down at concentrations above 1 μg/ml. As shown with the *ey-tTA* strain, tetracycline can be used to control the timing of induction at distinct phases of development in order to define a phenocritical period. Temporal control of gene expression should also be effective during pupal development as a function of the concentration of tetracycline provided to the larvae before pupation. They can be well synchronised during this developmental period and go through a number of well-characterised stages (Ashburner, 1989). In combination with the use of the *lacZ* reporter, these features should help in determining the time course of induction of any gene driven by a *tTA*-expressing line of interest.

Our attempts to use the reverse tetracycline-controlled transactivator (*rtTA*, Gossen et al., 1995) have been unsuccessful in *Drosophila*. This transactivator is based on a mutagenized version of *tTA*, which binds the *tetO* sequences only in the presence of specific tetracycline derivatives. It corresponds to a 4-amino-acid exchange in *tetR*, which is thought to alter the conformation of the repressor and allows its binding to DNA upon association with certain tetracycline compounds (Gossen et al., 1995). Since it was originally isolated in a genetic screen in bacteria and tested successfully in mammalian cells (Gossen et al., 1995) and in transgenic mice (Kistner et al., 1996), *rtTA* might need a temperature close to 37°C to be stable. In contrast, *tTA* shows a potent activity in *Drosophila* and its negative regulation by tetracycline is not a major difficulty, as described above. Furthermore, both the repression and the kinetics of gene induction might be

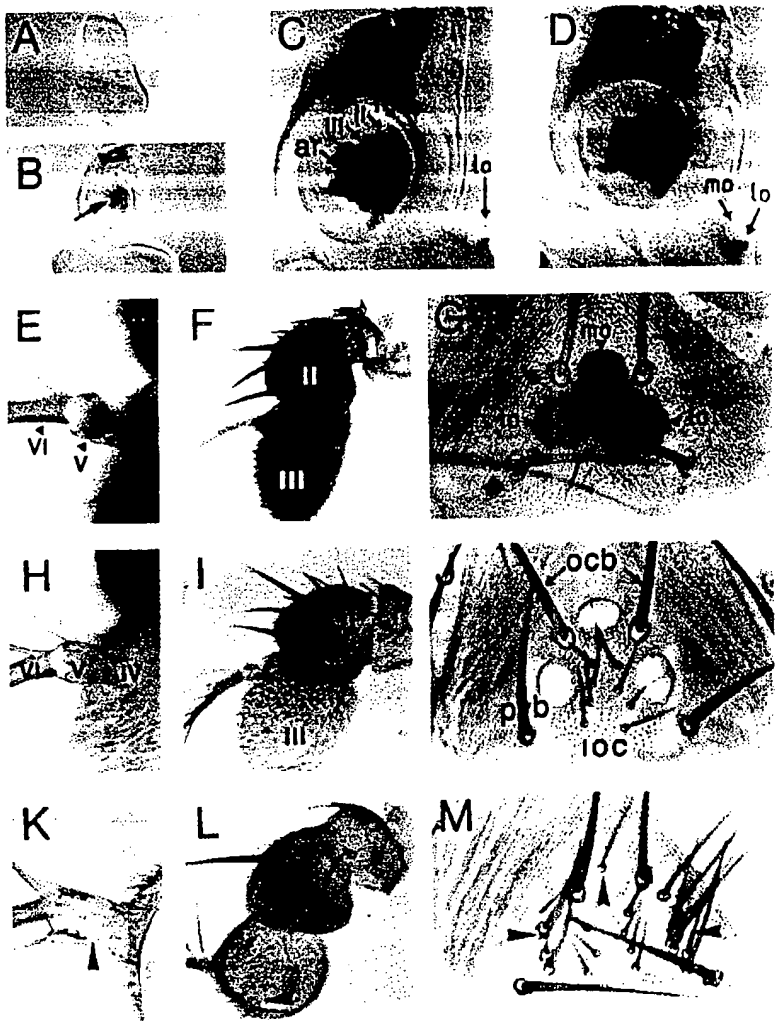

Fig. 5. Adult phenotype resulting from the directed expression of *Antennapedia* by the 205 strain. (A-D) Expression pattern of the 205 strain in the eye-antennal disc visualized by X-gal detection of β-galactosidase activity encoded by *tetO-lacZ* reporter. Discs are oriented with anterior up and dorsal left. (A) Early third instar. (B) Mid third instar; expression starts in the centralmost region of the antennal disc (arrow) and the presumptive palp region (arrowhead). (C) Late third instar larvae: expression has expanded in a wedge-shaped sector overlapping the most proximal region of the arista (ar) and the three major antennal segments (roman numerals). Expression in the lateral occellus (lo) that is not detectable in active wandering larvae is also indicated. (D) White prepupae: β-gal activity is detectable in the medium occellus (mo). (E-G) Expression of the *lacZ* reporter in the respective adult structures: the most proximal segments of the arista (E), the three antennal segments (F) and the occelli (G). (H-J) Normal phenotype of 205/+; *tetO-Antp*/+ adults raised continuously with 10 μg/ml tetracycline. Occipital, post vertical and interoccellar bristles are indicated by ocb, pvb and ioc, respectively. (K-M) Altered phenotype of 205/+, *tetO-Antp*/+ adults derived from larvae shifted from 10 μg/ml tetracycline to standard food during the late third instar. Note the thickening of the proximal segments of the arista (K, arrowhead), a bunch of new bristles on the third antennal segment and a modification in the number, the localization and the shape of the characteristic bristles of the second antennal segment (L, arrowheads). We could not determine the origin of these bristles on the basis of their morphology. Although different from the usual antennal bristles, they are not of a leg type because of the lack of the characteristic bract. The other main feature is a bunch of thick and long bristles of unknown origin close to the occelli (M, arrowheads).

increased further with one of the numerous tetracycline derivatives available. Some of them have been shown to be more potent effectors on *tTA* than tetracycline itself (Gossen and Bujard, 1993; Chrast-Balz and Hooft van Huijsduijnen, 1996).

Binary systems for controlling gene expression

The interest in using a binary system that combines an effector molecule for controlling activity of a responder promoter has been largely demonstrated with the GAL4/UAS system (Brand and Perrimon, 1993). Our method makes use of a similar experimental strategy, which allows the stable integration of any kind of construct in a parental fly strain in the absence of the transactivator. Tissue-specific activation of the gene construct is achieved in the offspring of a cross with a driver line chosen for its pattern of expression of the transactivator. The *tet* system provides a more versatile tool, owing to the possibility of controlling the timing of gene expression during development. In addition, the use of the vectors RHT and WTP facilitates the generation of driver strains expressing *tTA* under the control of previously isolated tissue-specific enhancers, and responder strains carrying any gene of interest under the regulation of the *tetO*-containing promoter. We also plan to generate a collection of *tTA*-expressing strains following the

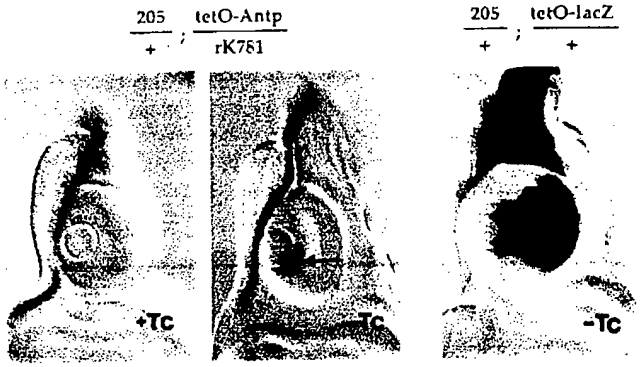

Fig. 6. Tetracycline-controlled expression of *Antennapedia* by the 205 strain activates rK781. Shown are β-galactosidase-stained antennal discs isolated from larvae raised continuously on 10 μg/ml tetracycline (+Tc) or shifted to standard food 48-72 hours before dissection (−Tc). The arrow points to the activation of rK781 in the centralmost region of the wedge-type sector of *tTA* expression, visualized on the right by the *tetO-lacZ* reporter. Eggs were collected over 12 hours from tetracycline-treated females of the genotype Cy[A405. M2]/205; rK781/rK781 mated with +/+ ; *tetO-Antp*/TM6,Tb males. Larvae of the genotype 205/+ ; *tetO-Antp*/rK781 were identified by their Tb$^+$ phenotype and the absence of the staining pattern due to the Cy[A405. M2] chromosome.

random integration into the genome of an enhancer detector construct with *tTA* as a reporter gene. The availability of strains expressing *tTA* in a wide variety of patterns will ensure a large number of applications for the *tet* system.

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APPENDIX F

In vivo analysis of scaffold-associated regions in *Drosophila*: a synthetic high-affinity SAR binding protein suppresses position effect variegation

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Scaffold-associated regions (SARs) were studied in *Drosophila melanogaster* by expressing a synthetic, high-affinity SAR-binding protein called MATH (multi-AT-hook), which consists of reiterated AT-hook peptide motifs; each motif is known to recognize a wide variety of short AT-rich sequences. MATH proteins were expressed specifically in the larval eye imaginal discs by means of the tetracycline-regulated transactivation system and tested for their effect on position effect variegation (PEV). MATH20, a highly potent SAR ligand consisting of 20 AT-hooks, was found to suppress *white*$^{mottled\ 4}$ variegation. This suppression required MATH20 expression at an early larval developmental stage. Our data suggest an involvement of the high AT-rich SARs in higher order chromatin structure and gene expression.

Keywords: chromatin structure/*Drosophila*/PEV/SAR

Introduction

Scaffold-associated regions (SARs), also called matrix attachment regions or MARs, are operationally defined as DNA sequences that specifically associate with the nuclear scaffold or matrix and possibly define the bases of chromatin loops (Mirkovitch *et al.*, 1984; Cockerill and Garrard, 1986; Gasser and Laemmli, 1986). SARs are very AT-rich regions of several hundred base pairs in length, that are possibly best described as being composed of numerous, irregularly spaced A tracts (short AT-rich sequences containing homopolymeric runs) (reviewed in Laemmli *et al.*, 1992). Proteins that specifically bind to SARs do not appear to interact with a precise base sequence but rather recognize certain structural features of non-B DNA, such as narrow minor grooves, DNA bends and a propensity to unwind (Bode *et al.*, 1992; Käs *et al.*, 1993).

SARs play roles in both chromosome condensation and gene expression. A recent report identified SARs as *cis*-elements of chromosome dynamics (Strick and Laemmli, 1995), while numerous publications demonstrated that SARs, in a flanking position, can strongly stimulate the expression of various heterologous reporter genes in different biological systems (Laemmli *et al.*, 1992). The SAR-mediated stimulation of transgene expression is not observed in transient assays, but only after stable integration of the test constructs into the genome (Klehr *et al.*, 1991; Poljak *et al.*, 1994). Hence, these *cis*-acting elements may exert their effect via chromatin structure, since transiently transfected DNAs are known to be poorly organized into nucleosomes.

A recent model attempts to explain the general stimulatory effect of SARs on transcription by proposing that SARs facilitate the displacement of histone H1 (a process referred to as chromatin opening) through mutually exclusive interactions with proteins of similar DNA-binding specificity, such as the high mobility group protein HMG-I/Y (Käs *et al.*, 1993). HMG-I/Y contains three short DNA-binding domains, called AT-hooks, that bind the minor groove of A tracts similarly to the peptide antibiotic distamycin (Reeves and Nissen, 1990). Histone H1, HMG-I/Y and distamycin all bind selectively to the A tracts of SARs, and competition experiments between them have demonstrated that HMG-I/Y and distamycin are 'dominant': i.e. both can displace pre-bound histone H1 from an SAR template (Zhao *et al.*, 1993). The observation that distamycin, added to cells, markedly stimulated cleavage at SARs by topoisomerase II in internucleosomal linker DNA but not at hypersensitive sites, lends *in vivo* support to this model; increased accessibility for cleavage presumably arises from the displacement of proteins, possibly histone H1 (Käs *et al.*, 1993). The model is also consistent with elegant studies demonstrating that SARs are necessary to spread open chromatin from enhancers to gene promoters (Kirillov *et al.*, 1996; Jenuwein *et al.*, 1997).

To study the role of SARs in chromosome condensation, synthetic multi-AT-hook proteins (MATH), consisting of numerous reiterated AT-hook peptide motifs derived from HMG-I/Y (Strick and Laemmli, 1995), were synthesized. Since the AT-rich SARs have enough adjacent binding sites to accommodate all the multiple, covalently linked AT-hooks, these HMG-I/Y derivatives bind SARs (both as DNA and chromatin) with exquisite specificity (Strick and Laemmli, 1995). Their effects on chromosome assembly were tested in *Xenopus* egg extracts capable of converting added nuclei to mitotic chromosomes *in vitro*. Remarkably, adding low levels of MATH20, a protein containing 20 hooks, inhibited the normal events of chromosome condensation, suggesting that SARs are *cis*-elements of mitotic chromosome dynamics (Strick and Laemmli, 1995).

To address the importance of SARs in the fruit fly *Drosophila melanogaster*, we expressed MATH20 in the larval eye imaginal disc to test for an effect on position effect variegation (PEV). We found that regulated expression of MATH20 led to a suppression of PEV, suggesting

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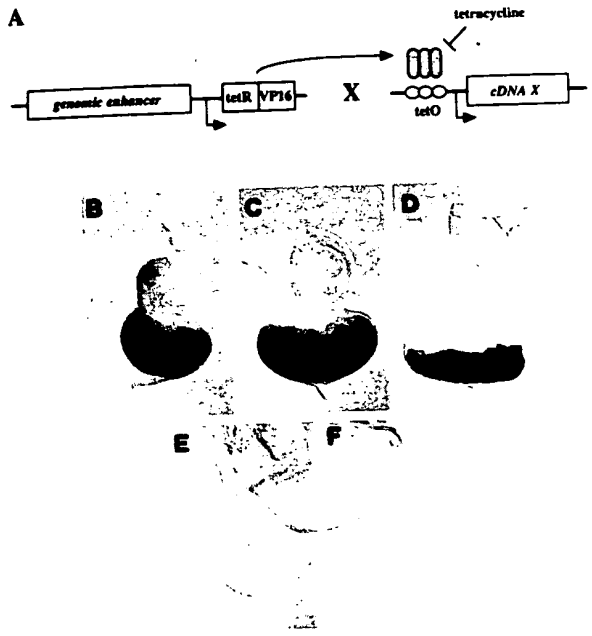

Fig. 1. Protein overexpression by means of the tetracycline-regulated transactivation system in *Drosophila*. (**A**) Schematic representation of the Tet system applied to *Drosophila* (see text for details). In (B–F), β-galactosidase staining of eye-antennae imaginal discs from early (**B** and **E**), mid (**C**) and late third instar larvae (**D** and **F**), showing the activation of a tetO–LacZ reporter construct by the *eyeless* enhancer-tTA strain. In (E) and (F), larvae were maintained on medium containing tet 0.2 μg/ml, resulting in a complete inhibition of the tTA-induced LacZ expression.

an involvement of SARs or SAR-like AT-rich regions in long-range chromatin structure and gene regulation.

Results

Targeted expression of synthetic SAR-binding proteins in Drosophila

With the aim of improving our understanding of SAR function(s) *in vivo*, we have overexpressed MATH20, the synthetic SAR-binding protein, in *Drosophila* by means of the tetracycline (tet)-regulated transactivation system (Tet system) (Bello *et al.*, 1998). Our strategy consisted of targeting expression of these proteins specifically to the eye imaginal discs during larval development and scoring for effects on *white* variegation. The principle of the binary Tet system is depicted in Figure 1A. A fly strain expressing a tet-regulated transactivating protein [tTA, a fusion of the tet repressor DNA-binding domain and the VP16 transcriptional activation domain (Gossen and Bujard, 1992)] under the control of regulatory sequences [in our case, an eye-specific enhancer identified in the intronic sequences of the *eyeless* gene (Quiring *et al.*, 1994)] is crossed to a strain containing the coding sequence for the gene of interest downstream of the tet operator sequences. The expression of this gene is then obtained in the progeny, in the same pattern as tTA is expressed, and can be tightly controlled by tet (Bello *et al.*, 1998). As shown in Figure 1B–D, by monitoring *tetO–LacZ* reporter gene expression, the *ey*-tTA strain can be used to target protein expression in the third instar eye imaginal discs, primarily in the undifferentiated eye cells (Figure 1B), and progressively only in the differentiated cells posterior to the morphogenetic furrow (Figure 1C and D). Additionally, β-galactosidase activity is also observed in the eye imaginal discs during the first and second instars (data not shown). Tet, when added to the food at a concentration as low as 0.2 μg/ml, is able to completely silence the expression of the *LacZ* reporter gene, both in early (Figure 1E) and late third instar (Figure 1F).

MATH20 suppresses PEV

In *Drosophila*, PEV refers to the mosaic expression of a gene when chromosome rearrangements place it close to heterochromatin. This heterochromatin-mediated gene silencing is proposed to be a heritable, epigenetic event that involves no alteration in the DNA content. Silenced genes are believed to be packaged into a higher order chromatin structure, or alternatively might be localized to a special nuclear compartment that confers transcriptional repression (reviewed in Karpen, 1994; Elgin, 1996). A classical example of PEV is the *white*-mottled (w^{m4}) inversion, in which the *white* gene necessary for the red pigmentation of the eye is juxtaposed close to heterochromatin of the X chromosome.

The variegating phenotype of *white*-mottled is seen as numerous clones of red, wild-type cells in an otherwise *white* mutant background (Figure 2A). Since MATH proteins are very efficient in SAR binding, including satellite III found in the centromeric heterochromatin of the X chromosome (Strick and Laemmli, 1995, and below), we reasoned that overexpressing MATH proteins specifically in the eye imaginal discs of the developing larvae might modify *white* variegation, a process known to involve chromatin structure. Females of the *ey*-tTA strain in a w^{m4} background were crossed to males of the various independent tetO-MATH20 strains. After eclosion, males of the desired genotypes (which are heterozygous for both *ey*-tTA and tetO-MATH20 transgenes, and hemizygous for w^{m4}) were kept for 5 days at 25°C and photographed. As a control, we used a line containing an empty tetO vector, showing a typical 'salt and pepper'-like mosaic expression of the *white* gene (Figure 2A). Expressing MATH20 resulted in a significant derepression of the *white* gene, and a general loss of the variegating phenotype. This suppression of PEV was clearly visible in various independent MATH20 lines (Figure 2B–F), and was shown to be highly reproducible. Quantitative analysis of the red eye pigment levels for seven independent MATH20-expressing strains is shown in Figure 3A, with PEV suppressor ratios ranging from 1.6 to 2.8 when compared with the control line (two independent control lines gave identical results). In contrast, MATH11, a less potent SAR DNA-binding protein (Strick and Laemmli, 1995), revealed no PEV-modifying effects in four independent strains (Figure 3A, dashed bars).

To test for the specificity of the MATH20-induced suppression of PEV, we made use of tet to silence the expression of the MATH20 transgene. Progeny of a cross between w^{m4}, *ey*-tTA females and tetO-MATH20-19 or empty tetO were grown on either normal or tet-containing food, under exactly the same conditions of temperature and population density as before. While tet treatment has no effect on the *white* mosaic expression in the control

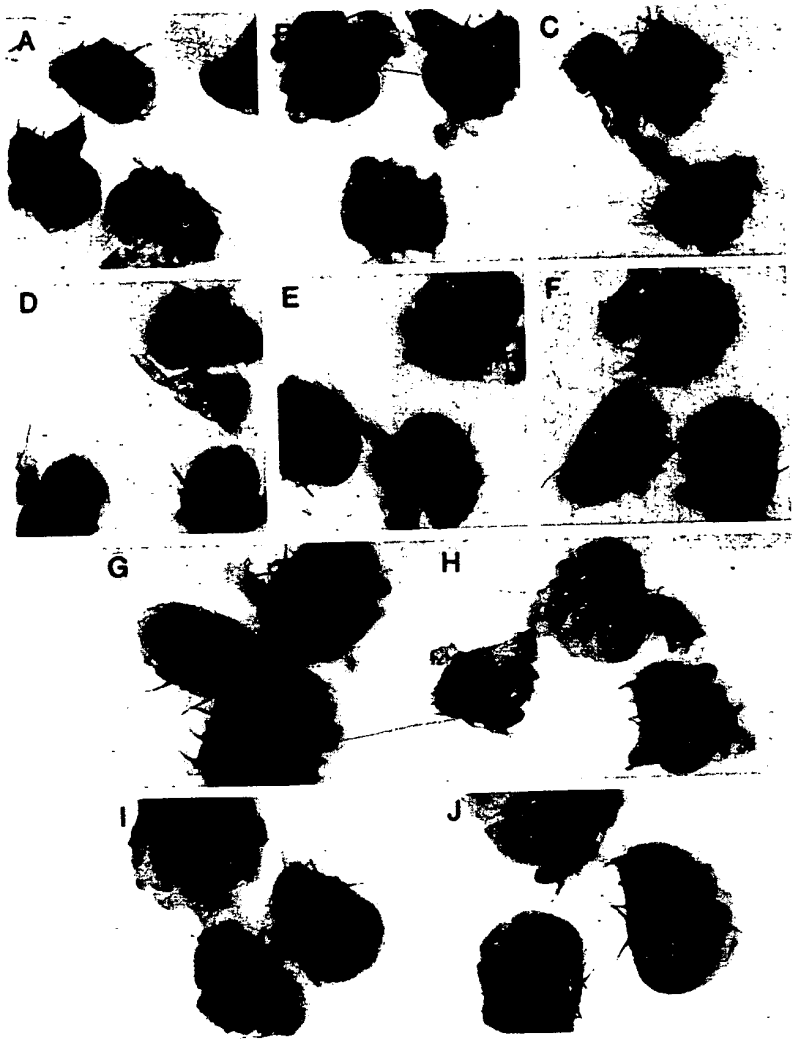

Fig. 2. Eye-specific expression of MATH20 suppresses *white* variegation. (A–F) Females of the *ey*-tTA strain in a w^{m4h} background were crossed to males of the various tetO-MATH20 strains. Progeny was grown on standard medium at 25°C. After adult eclosion, males of the desired genotype were kept for 5 days at 25°C before photography. Shown are photomicrographs of male heads of the following genotypes: (**A**) w^{m4h}/Y, control tetO/+, *ey*-tTA/+; (**B**, **E** and **F**) w^{m4h}/Y, tetO-MATH20/+, *ey*-tTA/+, with respectively strains 20-14, 20-2 and 20-19. (**C** and **D**) w^{m4h}/Y, +/+, *ey*-tTA/tetO-MATH20, with respectively strains 20-4 and 20-6. (G–J) Inhibition of MATH20-induced PEV suppressor effect by tet treatment. Females of the *ey*-tTA strain in a w^{m4h} background were crossed to males of the control tetO strain (**G** and **H**) or the tetO-MATH20-19 strain (**I** and **J**). Progeny was grown at 25°C, either on standard medium (G and I) or medium containing tet 0.2 μg/ml (H and J).

line (Figure 2G–H), it clearly inhibits MATH20-induced suppression of PEV (compare Figure 2I and J). Quantitative analysis is given for three MATH20 strains (Figure 3B). Results are shown as the ratio of OD_{480} MATH20 to OD_{480} control: while modification of *white* variegation is observed in the absence of tet (Figure 3B, open bars), tet treatment, by maintaining silent the MATH20 transgene expression, leads to eye pigment levels very similar to those of the control (Figure 3B, black bars).

We next made use of tet to examine whether suppression of PEV by MATH20 might require expression during an early developmental stage. For this purpose, 50 females of the w^{m4h}, *ey*-tTA strain were crossed to males of the control or tetO-MATH20-12 strain. One hour egg collections were made, and kept on either normal or tet-containing food. Larvae were then transferred at regular intervals to normal food. In these conditions, the transgene is kept efficiently silent, but it is activated after shifting larvae off tet following a lag period of ~12 h (Bello *et al.*, 1998). As shown in Figure 3C, PEV suppression is still observed when larvae are exposed to tet up to 60 h after egg laying (AEL). If the gene is kept silenced longer (72–120 h), then no PEV suppression is observed. The *eyeless* enhancer activity and hence MATH20 expression can be detected throughout the larval stages and up through early pupal stages when differentiated eye cells develop from precursor cells. Since activation of the transgene, during the third instar period (72 h AEL), no longer reduced PEV, we conclude that MATH20 expression in the undifferentiated cells is required to achieve suppression of PEV.

MATH20 suppresses cleavage by topoisomerase II in satellite III heterochromatin of chromosome X

Is suppression of PEV by MATH20 mediated by interactions at SARs? The w^{m4} inversion juxtaposes the *white* gene to the heterochromatin of the X chromosome. Intriguingly, the predominant component of the

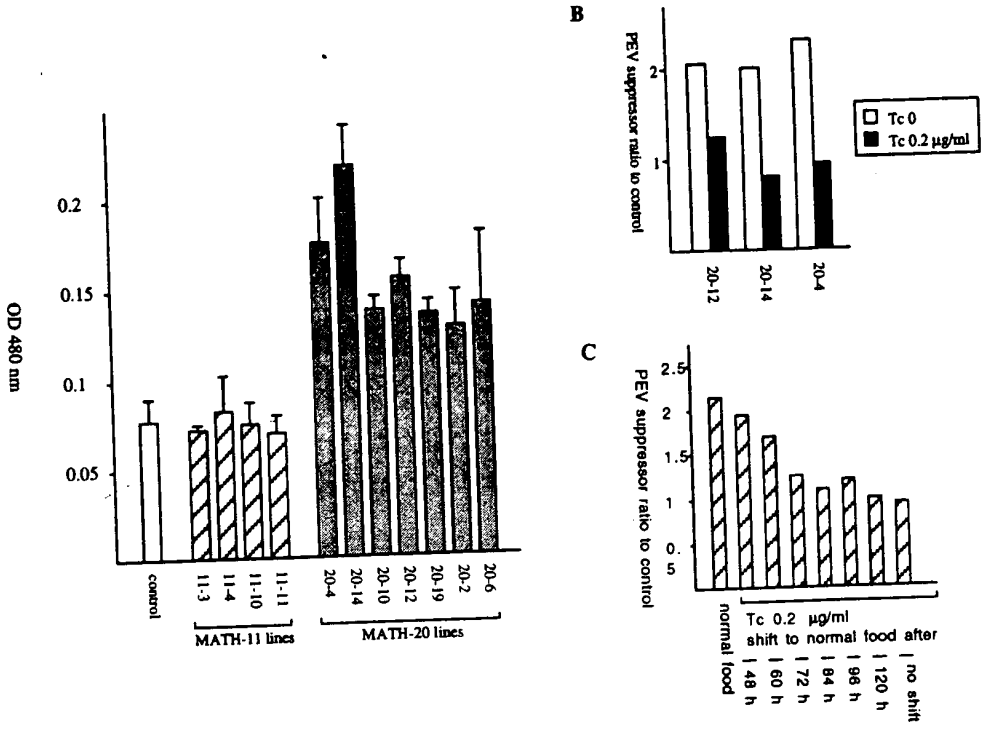

Fig. 3. Quantitation of MATH20-induced PEV suppression. (**A**) MATH20, but not MATH11, suppresses w^{m4} variegation. Females of the *ey*-tTA strain in a w^{m4h} background were crossed to males of the control tetO strain or the various tetO-MATH11 (dashed bars) and tetO-MATH20 strains (shaded bars). Quantitation of the red eye pigment levels was done on groups of 40 male heads 5 days after eclosion, and repeated 4–6 times. Standard deviations are shown as thin lines above the histograms. (**B**) Inhibition of MATH20-induced PEV suppression by tet. Females of the *ey*-tTA strain in a w^{m4h} background were crossed to males of the control tetO strain or three independent tetO-MATH20 strains. Progeny were grown at 25°C on either normal food (open bars) or food containing tet 0.2 µg/ml (black bars). Values are given as the ratio of the $OD_{480\ nm}$ of the tetO-MATH20 lines to the $OD_{480\ nm}$ of the empty tetO vector control line, and represent the average of three independent measurements. (**C**) Females of the *ey*-tTA strain in a w^{m4h} background were crossed to males of the control tetO strain or tetO-MATH20-12 strain. Progeny were kept on normal or tet-containing food (0.2 µg/ml). Larvae were transferred to normal food at the indicated times after egg laying. The red eye pigment levels were measured in groups of 30 male heads, and repeated three times. Values are given as the average ratio of the $OD_{480\ nm}$ of the tetO-MATH20-12 strain to the $OD_{480\ nm}$ of the control strain.

heterochromatin is satellite III, also called the 1.688 or the 359 bp repeat satellite (Hsieh and Brutlag, 1979). Two or three repeats (718–1017 bp) of this satellite behave as fully fledged SARs *in vitro*; they preferentially bind nuclear scaffolds, topoisomerase II and HMG-I/Y (Käs and Laemmli, 1992; Karpen, 1994). Thus, the inverted *white* gene appears juxtaposed to a giant, reiterated SAR of ~11 Mb.

Topoisomerase II is one of the growing number of proteins associated with heterochromatin (Rattner *et al.*, 1996). Although it is not known whether topoisomerase II is implicated in heterochromatin formation, it is one of the few proteins for which the site of interaction can be studied by stabilizing the so-called cleavage intermediate using cytotoxic drugs. Thus, this protein serves here as a convenient, heterochromatin-associated reporter protein which is expected to monitor the interaction of MATH20 in this chromatin. Moreover, topoisomerase II is known to be specifically enriched over satellite III heterochromatin, as revealed by microinjection of fluorescent topoisomerase II into *Drosophila* embryos (Denburg *et al.*, 1996). This preferential interaction was also borne out by previous studies that demonstrated a major topoisomerase II cleavage site once per satellite III repeat; this 359 bp repeat contains two positioned nucleosomes, and the major topoisomerase II cleavage site occurs in one of two nucleosomal linker regions as depicted in Figure 4B.

Does MATH20 interfere with topoisomerase II cleavage in satellite III? We addressed this question using *Xenopus* egg extracts. These extracts are known to carry out faithfully many cellular processes. Indeed, we noted that the endogenous topoisomerase II of such extracts generated a cleavage ladder in satellite III repeats of *Drosophila* Kc nuclei that is indistinguishable from the one observed in cells (Figure 4A, lanes 1 and 2). Interestingly, this cleavage ladder is suppressed specifically in a dose-dependent manner by MATH20 (lanes 2–5). In contrast, no inhibition is observed by added HMG-I/Y (lane 6) which binds much more dispersively to the genome. In conclusion, MATH20 can interfere specifically with topoisomerase II cleavage in satellite III, strongly suggesting that the MATH20 protein encoded by the transgene expressed in flies is very likely to bind specifically the heterochromatin of chromosome X.

Discussion

The variegated phenotype of *white* mottled flies is the result of a large inversion in the X chromosome that places the *white* gene adjacent to centomeric heterochromatin. PEV is due to a stochastic inactivation of the *white* gene in some but not other cells at an early stage of eye development, followed by clonal maintenance through later stages. The resulting pattern of *white* gene expression

Fig. 4. Specific suppression by MATH20 of topoisomerase II cleavage in satellite III of chromosome X. This figure demonstrates that MATH20 specifically inhibits topoisomerase II cleavage in satellite III, which is the predominant component of the heterochromatin of chromosome X. (**A**) Isolated Kc *Drosophila* nuclei were incubated in mitotic *Xenopus* egg extracts in the presence of different concentrations of MATH20 or HMG-I/Y. The topoisomerase II cleavage activity subsequently was monitored in satellite III by treating the extracts for 10 min with VM26. The DNA samples were displayed on a 1.2% agarose gel and the Southern blot hybridized with a satellite III repeat probe. VM26 and proteins were added as indicated at the top. The sample in lane 1 received no VM26 and no MATH20, and that in lane 2 received VM26 only. Samples in lanes 3–5 contained 80, 40 and 20 ng of MATH20, respectively. The sample in lane 6 contained 80 ng of HMG-I/Y. (**B**) The repeat structure of satellite III chromatin, which consists of two nucleosomes per 359 bp repeat unit. Topoisomerase II cleaves (arrow) once per unit, in every other nucleosomal linker region (Käs *et al.*, 1993).

is observed in the eye as patches of pigmentation. Morphologically, PEV is observed on polytene chromosomes as a spreading of heterochromatic structures into euchromatic genes (Elgin, 1996). Current evidence favors a model according to which silencing proteins of the centromeric heterochromatin spread into the juxtaposed euchromatic region by a cooperative assembly process. This spreading may be a consequence of the inversion by removing putative boundary elements that otherwise delimit heterochromatin (Locke *et al.*, 1988).

We have shown here that specific expression of the artificial high-affinity SAR-binding protein MATH20 in the developing eye imaginal discs results in suppression of *white* variegation. Using the tetracycline gene regulation system, we demonstrated that suppression of PEV requires the expression of MATH20 in the undifferentiated eye cells and is observed if this transgene is activated up to 60 h after egg laying. In contrast, no suppression is observed upon activation of MATH20 during the late third instar period. Interestingly, suppression of PEV by MATH20 was also observed for the *Brown*^{D} variegating rearrangement (data not shown). We found no effect on *white* variegation by expression of MATH11; this protein with only 11 AT-hooks has a 7-fold lower binding affinity (K_D = 18.2 pM) for SARs than MATH20 (2.6 pM). Consequently, proportionally higher amounts of protein were required to affect chromosome condensation in *Xenopus* extracts (Strick and Laemmli, 1995). Hence, it might be necessary to express MATH11 in the eye imaginal disc with a stronger promoter to achieve an effect on *white* variegation. The differential effect of MATH11 versus MATH20 underscores the notion that suppression of PEV is a specific phenomenon; it appears to be related to the binding strength of the effector. Adding MATH to a mitotic *Xenopus* extract led to the formation of abortive condensation products (Strick and Laemmli, 1995), and microinjection of MATH20 (but not HMG-I/Y) blocked HeLa cells in late G_2-phase following passage through S-phase (R.Strick, R.Peperkok and U.K.Laemmli, in preparation). In agreement with this, we have observed that higher levels of embryonic and larval expression of either MATH20 or MATH11 led to lethality. Thus, suppression of PEV required tissue-specific expression of MATH20 at a low level that does not interfere with cell division.

The inverted *white* gene is juxtaposed to a giant 11 Mb reiterated SAR in the form of satellite III repeats. MATH20 binds there with great specificity (Strick and Laemmli, 1995) and is able to interfere with the activity of topoisomerase II (Figure 4). This observation establishes proof of the principle that MATH20 can interact with or displace a pre-bound protein associated with heterochromatin. Although we cannot rule out other possible models, it is tempting to explain the effect of SAR on gene expression, chromatin opening and PEV by extending a model put forward by Laemmli and colleagues (Laemmli *et al.*, 1992). In this model, certain proteins (called compacting proteins here) interact with SARs or certain AT-rich satellites cooperatively; this can lead to either chromatin folding, chromosome condensation (looping) or formation of heterochromatin. Conversely, other proteins such as HMG-I/Y and their monster derivatives, MATH, bind non-cooperatively to SARs and can displace the compacting proteins by disrupting their cooperative interactions, hence resulting in chromatin unpacking. These alternative chromatin states are governed by the binding strength and the relative local level of the chromatin packaging proteins versus those that undo it. Of importance for these considerations is the extent of SAR repetition at a given region. Since an assembly of cooperatively interacting proteins becomes energetically more favorable with increasing repeats, certain packaging proteins are expected to polymerize preferentially onto satellite III chromatin over individual SARs. In contrast, the non-cooperative MATH

proteins would bind dispersively to single and reiterated SARs. Thus in a cell, while a single SAR in euchromatin may promote chromatin opening and stimulation of gene expression (Jenuwein *et al.*, 1997), a reiterated SAR could result in silencing.

It is easy to explain the effect of MATH20 on PEV by simple considerations based on the above model. The high affinity of MATH20 for SARs could allow it to bind to the satellite III region or other AT-rich regions, thus disrupting the cooperative interaction of the compacting proteins. This in turn would energetically disfavor the spreading of the polymerizing proteins into the flanking euchromatic region. As the probability of inactivating an adjacent euchromatic gene diminishes, one expects to observe suppression of PEV. It is impossible at present to obtain direct evidence for the model, and for a direct *in vivo* proof for MATH20 binding to satellite III. PEV appears to be a complex, poorly understood process, and the nature of the *cis*-DNA elements involved in heterochromatin formation has yet to be elucidated. The results reported here could also provide a clue about the nature of these *cis*-acting elements involved in PEV; the data suggest that this chromatin state might be mediated in part by proteins that interact with AT-rich repeats, such as those of satellite III.

Materials and methods

Fly strains

The yw^{67c23} strain was used as recipient for injections. P-element-mediated germ line transformation was done using standard procedures (Spradling and Rubin, 1982a,b). For each construct, multiple independent lines were established, and the chromosomal location of the inserted transgene was determined by standard genetic analysis using balancer chromosomes. The $white^{mottled\ 4}$ inversion, $In(1)w^{m4h}$ strain, was used to score the effects of MATH proteins on *white* variegation. PEV-modifying effects were quantified by red eye pigment measurement (Ashburner, 1989), on groups of 40 male heads of the desired genotype, kept for 5 days at 25°C after eclosion. Flies were grown at 25°C on standard medium, unless specified otherwise in the text. Tetracycline (Sigma) was used at 0.2 μg/ml as described (Bello *et al.*, 1998). Fly strains expressing tet-VP16 transactivator are described elsewhere (Bello *et al.*, 1998).

Plasmid constructs

Further details of the cloning procedures are available upon request. MATH11 and MATH20 cDNAs (Strick and Laemmli, 1995) were inserted into pWTP (marked with mini*white*) (Bello *et al.*, 1998) and/or pYTP (marked with *yellow*). pYTP was constructed by inserting a tet operator–P promoter–SV40 polyadenylation signal cassette from pWTP into pY vector, made by inserting the *yellow* gene from the YES vector (Patton *et al.*, 1992) into Carnegie 4 (Rubin and Spradling, 1983).

β-Galactosidase activity

β-Gal stainings were done as described (Ashburner, 1989), on glutaraldehyde-fixed eye-antennal imaginal discs from early and late third instar larvae.

Topoisomerase II cleavage assay

Kc nuclei were isolated (Mirkovitch *et al.*, 1984) and added to *Xenopus* egg extracts (Strick and Laemmli, 1995) to a final concentration of 10 000 nuclei/μl of extract, incubated at 21°C for 1 h and subsequently treated with 50 μM VM26 for 10 min. The reactions were stopped and isolated DNA samples were electrophoresed in a 1.2% agarose gel, electroblotted to nylon membranes and hybridized to satellite III repeat probes as described (Käs *et al.*, 1993).

Acknowledgements

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ORIGINAL PAPER

APPENDIX G

E. T. Bieschke · J. C. Wheeler · J. Tower

Doxycycline-induced transgene expression during Drosophila development and aging

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Abstract The "reverse" tetracycline repressor (rtR) binds a specific DNA element, the tetracycline operator (tetO), only in the presence of tetracycline, or derivatives such as doxycycline (dox). Fusion of rtR to the transcriptional activation domain of herpes virus protein VP16 produces a eukaryotic transactivator protein (rtTA). rtTA has previously been shown to allow dox-dependent transcription of transgenes linked to tetO sequences in mammals. To adapt this system to *Drosophila*, the *Actin5C* promoter was used to drive constitutive expression of rtTA in transgenic flies. Three reporter constructs, each encoding *E. coli* β-galactosidase (β-gal), were also introduced into transgenic flies. In one reporter seven tetO sequences were fused to the *Adh* core promoter. The other two reporter constructs contain seven tetO sequences fused to the *hsp70* core promoter. Feeding of transgenic *Drosophila* containing the rtTA construct and any one of the three reporter constructs with dox caused up to 100-fold induction of β-gal. Dox induced β-gal expression in all tissues, in larvae and in young and senescent adults. Induction of β-gal in adults had no detectable effect on life span. These results suggest the potential usefulness of this system for testing specific genes for effects on *Drosophila* development and aging.

Key words Tetracycline repressor · Inducible promoter · Drosophila · Aging

Introduction

Inducible gene expression systems have long been an important tool in analyzing the function of specific genes in bacteria, yeasts, and *Drosophila*. In *Drosophila*, inducible transgenic systems usually rely wholly or in part on the use of a heat shock protein (hsp) gene promoter, which is transcriptionally induced in response to heat stress (Lis et al. 1983). While hsp gene promoters have been used to great advantage in many experiments, the system has several important limitations. First, the heat stress required for induction can have pleiotropic effects, including developmental abnormalities (phenocopies) (Lindquist 1986) and reduced fertility and viability. This is a problem particularly in experiments designed to study the aging process, since life span will be dramatically affected by changes in fertility and viability (Tower 1996). Another situation where the use of a heat-inducible promoter is problematic is the analysis of the heat shock proteins themselves. It is not possible to induce expression of a single hsp and study its effects, without the complication of inducing the entire endogenous repertoire of hsps. This problem has been overcome in certain experiments by using the metallothionein promoter (Petersen and Lindquist 1988; Solomon et al. 1991), which is inducible by heavy metal ions. However, the metallothionein promoter functions only in specific gut cells in transgenic *Drosophila* (Otto et al. 1987), thus limiting its potential usefulness. Therefore, there is a need for an alternative inducible gene expression system in *Drosophila*.

In the last five years, efficient inducible gene expression systems have been developed for mammalian systems based on the *E. coli* tetracycline repressor (tetR) (Gossen and Bujard 1992; Furth et al. 1994). The tetR binds to its target sequence, the tetracycline operator (tetO) only in the absence of the antibiotic tetracycline. The first system developed for mammals was the "tet-off" system (Gossen and Bujard 1992; Furth et al. 1994; Shockett et al. 1995). tetR protein was fused with the transcriptional activation domain of herpes virus transcription factor VP16. In the absence of tetracycline this protein binds to tetO sequences placed within the promoter of a gene of interest, thereby driving transcription. Addition of tetracycline then prevents binding and stops transcription ("tet-off"). A "tet-on" system was created

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by generating a mutant tetR:VP16 fusion protein, which had the reverse property of only binding to the tetO and activating transcription in the presence of tetracycline ("tet-on") (Gossen et al. 1995; Kistner et al. 1996). We report here the successful adaptation of the "tet-on" system to transgenic *Drosophila*.

Materials and methods

Plasmid constructions

Plasmid rtTA (reverse-tetracycline Trans Activator) was constructed by first inserting the 850-bp *Hind*III-*Xba*I fragment from pCaSpeR-AUG/β-gal (Thummel et al. 1988), containing the SV40 splice and poly(A) signals, into the *Hin*dIII (partial restriction digestion) and *Xba*I sites of the polylinker of the pCaSpeR4 transformation vector (Thummel and Pirotta 1992), to generate plasmid cSV. Plasmid pUHD172–1neo (Gossen et al. 1995) was digested with *Eco*RI, endfilled with T4 polymerase, then digested with *Bam*HI, to liberate a 1-kb fragment containing the reverse-tetracycline trans-activator coding sequence. Plasmid cSV was digested with *Spe*I, endfilled with T4 polymerase, then digested with *Bam*HI, and the 1-kb fragment from pUHD172–1neo was inserted, to generate the plasmid cTSV. DNA sequencing of cTSV revealed that it had resulted from an unexpected ligation event: the *Eco*RI site from the inserted fragment was conserved in this cloning step, and the 1-kb fragment was actually inserted into the *Bam*HI site, without any change in the *Spe*I site. The *Actin5C* promoter was inserted into plasmid cTSV in several steps. First, plasmid D237 (also called "Act5C > Draf+ > nuc-lacZ"; Struhl and Basler 1993) was digested with *Not*I, endfilled with T4 polymerase, then digested with *Kpn*I, and the resultant 4.3-kb fragment containing the *Actin5C* promoter was inserted into the *Kpn*I/*Eco*RV sites of pBlueScript II KS (Stratagene), to generate pAc. The 4.3-kb *Actin5C* promoter fragment was liberated from pAc by restriction digestion with *Kpn*I and *Eco*RI, and inserted into the *Kpn*I/*Eco*RI sites of cTSV, to generate the plasmid cATSV. DNA sequencing revealed that the *Actin5C* promoter in plasmid cATSV was in the wrong orientation relative to the reverse-tetracycline transactivator coding region. To correct this, the *Actin5C* promoter region was liberated by digestion with *Eco*RI, and then re-inserted into the same *Eco*RI site. DNA sequencing was used to identify a construct with the *Actin5C* promoter in the correct orientation, which was then named plasmid rtTA.

The seven tandem repeats of the tetO region in plasmid pUHC13–3 (Gossen et al. 1995) were amplified by PCR using the primers: 5′-TCGACTGCAGCTTTCGTCTTCAAGAATTCCTC-GAG-3′ and 5′-AGCTTCTAGATACACGCCTACTCGACCC-GGGTACCGAG-3′. The 367-bp PCR product was digested with *Pst*I and *Xba*I at the sites engineered into the primers, and then inserted into the *Pst*I/*Xba*I sites of pBlueScript II, to generate plasmid p7T.

Plasmid 7TAdh was constructed as follows. Plasmid pAdh/β-gal (Irvine et al. 1991; Koelle et al. 1991) was partially digested with *Eco*RI, and then completely digested with *Pst*I to liberate a 4.8-kb fragment containing the *Adh* basal promoter region (positions −33 to +53), the *Ubx* 5′ leader sequences fused to *lacZ*, and the SV40 splice and poly(A) signals. This fragment was cloned into the *Pst*I/*Eco*RI sites of the pCaSpeR4 polylinker, to generate plasmid pCaSpeR-Adh/β-gal. The 359-bp *Pst*I-*Xba*I fragment from plasmid p7T, containing the heptameric tetO region, was then inserted into the *Pst*I/*Xba*I sites of pCaSpeR-Adh/β-gal, to generate plasmid 7TAdh.

Plasmid 7T40 was constructed as follows. Construct c70Z (Simon and Lis 1987) was digested with *Hin*dIII and *Eco*RI to liberate a fragment containing the *hsp70* promoter fused to *E. coli lacZ*. This *Hin*dIII-*Eco*RI fragment was cloned into the *Hin*dIII/*Eco*RI sites of plasmid pBS2N to generate plasmid pBS2N′. Plasmid pBS2N is pBlueScript II KS + (Stratagene) in which the unique *Kpn*I site has been converted to a *Not*I site (a gift of L.R. Bell, University of Southern California). Construct c70Z was also digested with *Eco*RI alone to liberate an *Eco*RI fragment containing the *hsp70* poly(A) signal sequences, and this fragment was cloned into the unique *Eco*RI site of plasmid pBS2N′ to generate plasmid pBS2N″. Plasmid pBS2N″ was digested with *Hin*dIII and *Apa*I, treated with exonuclease III and with nuclease S1, and then ligated. The resultant plasmid was called c40Z, and DNA sequencing revealed a 5′ *hsp70* promoter deletion to position −40 relative to the start site of transcription. Plasmid c40Z is one of a series of *hsp70* 5′ promoter deletions which will be described in detail elsewhere (J. C. Wheeler and J. Tower, unpublished data). Plasmid c40Z was digested with *Not*I to liberate a 3.7-kb fragment containing the entire 5′Δ-40 *hsp70:lacZ* fusion gene, and this fragment was cloned into the *Not*I site of p7T, to generate plasmid p7T40-pre. A fragment containing the seven tetO repeats and the entire 5′Δ-40 *hsp70:lacZ* fusion gene was liberated from p7T40-pre by digestion with *Xho*I and *Spe*I, then inserted into the *Xho*I/*Spe*I sites in the polylinker of pCaSpeR4, to generate plasmid 7T40.

Plasmid 7TAUG was constructed as follows. A 4.6-kb SalI fragment from pCaSpeR-AUG/β-gal (Thummel et al. 1988), containing the *Adh* translation initiation sequence fused to *lacZ* and the SV40 splice and poly(A) signals, was cloned into the *Sal*I site of pBlueScript II KS, to generate plasmid pAUG. A *Pst*I fragment from plasmid 7T40, containing the seven tetO repeats and the *hsp70* promoter from −40 to +86, was inserted into the *Pst*I site of pAUG, to generate plasmid p7TAUG. A fragment containing the seven tetO repeats and the entire *hsp70:lacZ* fusion gene was liberated from p7TAUG by digestion with *Xho*I, and inserted into the *Xho*I site of pCaSpeR4, to generate plasmid 7TAUG.

Drosophila culture

Fly stocks were maintained on cornmeal/agar medium (Ashburner 1989). To obtain adult flies of defined ages, stocks were cultured at 25°C until 0–2 days post-eclosion, and then males only were transferred to 25°C or 29°C as indicated in Figure legends. These males were maintained at < 50 per vial and transferred to fresh vials every 2–4 days. Double transgenic adult males were obtained by crossing males of a transactivator stock (rtTA) to virgins of the reporter stocks (7TAdh, 7T40, and 7TAUG). Transgenic flies were generated by standard methods (Rubin and Spradling 1982), using the w^{1118} recipient strain.

Doxycycline treatments

Young flies (5–7 days post-eclosion) and old flies (28–32 days post-eclosion) were treated with the tetracycline derivative doxycycline hydrochloride (dox) (Sigma) by feeding. The indicated concentration of dox, in 20 mM Tris (pH 7.5) containing 10% sucrose, was soaked into a single Kim-Wipe (Kimberly-Clark), in an empty *Drosophila* culture vial. After feeding with dox for the specified time, the flies were returned to cornmeal/agar food vials, and allowed to recover as indicated. For treatment of larvae, the cornmeal/agar medium was supplemented with dox to a final concentration of 0.25 mg ml, prior to seeding of the culture.

Spectrophotometric assay of β-galactosidase activity

β-Galactosidase (β-gal) activity was quantitated in whole fly extracts using published procedures (Simon and Lis 1987). Assays were performed under conditions in which the reaction was linear with regard to the amount of extract. Data are presented as the average +/− the standard deviation for triplicate assays. Protein concentration of extracts was determined using the Bradford reagent (BioRad). The w^{1118} strain was used to generate all transgenic lines, and no β-gal activity was detectable in extracts of the w^{1118} strain using the spectrophotometric assay.

In situ staining for β-galactosidase activity

β-galactosidase expression was visualized in dissected flies, larvae, and cryostat sections using published procedures (Simon et al. 1985).

Results

Basic components of the system

To achieve tetracycline-inducible induction of transgenes in all tissues it is necessary that the reverse tetracycline transactivator (rtTA) be expressed in all tissues. The rtTA is a fusion of the reverse tetracycline receptor (rtR), which binds to DNA only in the presence of tetracycline, with the transcriptional activation domain of herpes virus transcription factor VP16. In construct rtTA, the constitutive *Drosophila Actin5C* promoter was used to drive expression of the rtTA coding region. This construct also contains the SV40 poly(A) signal sequence (Fig. 1A). To test the system, three reporter constructs were generated, each encoding *E. coli* β-gal. The constructs differed in the source of the core promoter, 5′ UTR, and polyadenylation signal sequences in order to maximize the chances of generating a construct which could yield high-level transgenic protein expression in *Drosophila*. In the first reporter construct (7TAdh) seven tetO sequences are fused to the *Adh* core promoter, followed by the *Ubx* 5′ untranslated region, the *E. coli lacZ* coding region, and the SV40 poly(A) signal (Fig. 1B). A regulatory element composed of seven tetO sequences was chosen because this element was previously shown to function in transgenic mice (Kistner et al. 1996). In the second reporter (7T40), the seven tetO sequences are fused to the *hsp70* core promoter, and *hsp70* 5′ untranslated region, followed by the *E. coli lacZ* coding sequences and the *hsp70* poly(A) signal (Fig. 1C). In the third construct (7TAUG), the seven tetO sequences are fused to the *hsp70* core promoter, followed by the *Adh* 5′ untranslated region, the *lacZ* coding region, and the SV40 poly(A) signal (Fig. 1D). Multiple independent transgenic lines were generated for each construct. Each line is homozygous for the transgenic construct, and is designated by the name of the construct followed by the chromosome in which the construct is inserted (in parenthesis), followed by a letter/number combination for each independent transgenic line. For example, line 7TAdh(2)A2 is transgenic line number A2 and has the 7TAdh construct inserted on the second chromosome.

Flies were then generated which contained both the rtTA construct and the 7TAdh construct ("double-transgenic" flies). This was done by crossing flies of stock rtTA(2)C1 to flies of stock 7TAdh(2)A2, which yields progeny containing one copy of each construct. A sample of these double transgenic flies were fed sucrose solution containing 1.0 mg/ml dox for 48 h, while the controls were fed sucrose solution alone. The flies were allowed to recover for 3 days, then sectioned using a cryostat, and the sections were stained for β-gal activity (Fig. 2A). In the treated flies robust β-gal activity (blue stain) was detected in all tissues. In the control flies, low-level β-gal activity was detected primarily in the gut, and thus the system allows dox-induced transgene expression in all tissues of the adult. The same results were obtained with transgenic flies containing the other two reporter constructs, 7T40 and 7TAUG (data not shown).

To determine if the system also works during development, line rtTA(2)C1 was crossed again to reporter line 7TAdh(2)A2, and also to reporter line 7T40(3)B1, and the larvae from each cross were cultured on food containing 0.25 mg/ml dox, and on

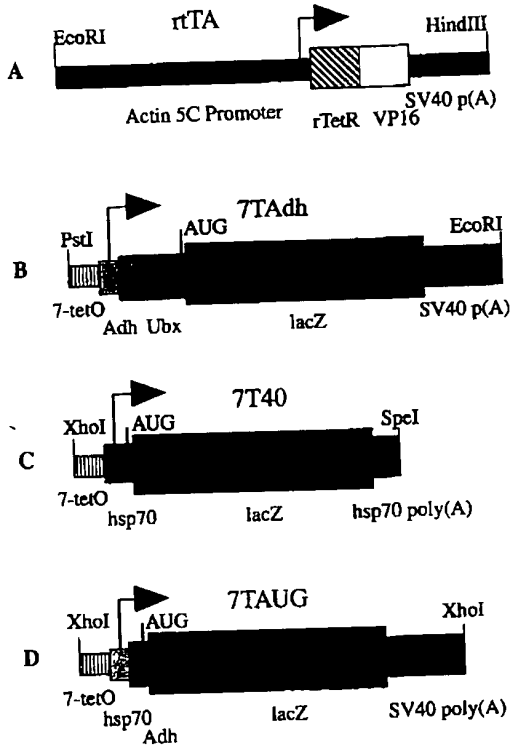

Fig. 1A–D Transgenic constructs. Each construct fragment shown is cloned into the indicated restriction sites of the polylinker of the pCaSpeR-4 transformation vector. The assembly of each construct is described in detail in Materials and methods. Diagrams are not to scale. **A** rtTA. The constitutive *Actin5C* promoter and 5′ untranslated region are fused to the coding sequences for the rtTA (reverse tetracycline transactivator), which is a fusion of the rtR (reverse tetracycline repressor) and the transcriptional activation domain of herpes virus protein VP16. The poly(A) signal sequences are from SV40. **B** 7TAdh. Reporter construct consisting of seven tetO sequences, the *Adh* core promoter, the *Ubx* 5′ untranslated region and translational initiation sequence, the *E. coli lacZ* coding region and the SV40 poly(A) signal sequences. **C** 7T40. Reporter construct consisting of seven tetO sequences, the *hsp70* core promoter, 5′ untranslated region and translational initiation sequence, the *E. coli lacZ* coding region and the *hsp70* poly(A) signal sequence. **D** 7TAUG. Reporter construct consisting of seven tetO sequences, the *hsp70* core promoter, the *Adh* 5′ untranslated region and translational initiation sequence, the *E. coli lacZ* coding region, and the SV40 poly(A) signal sequence

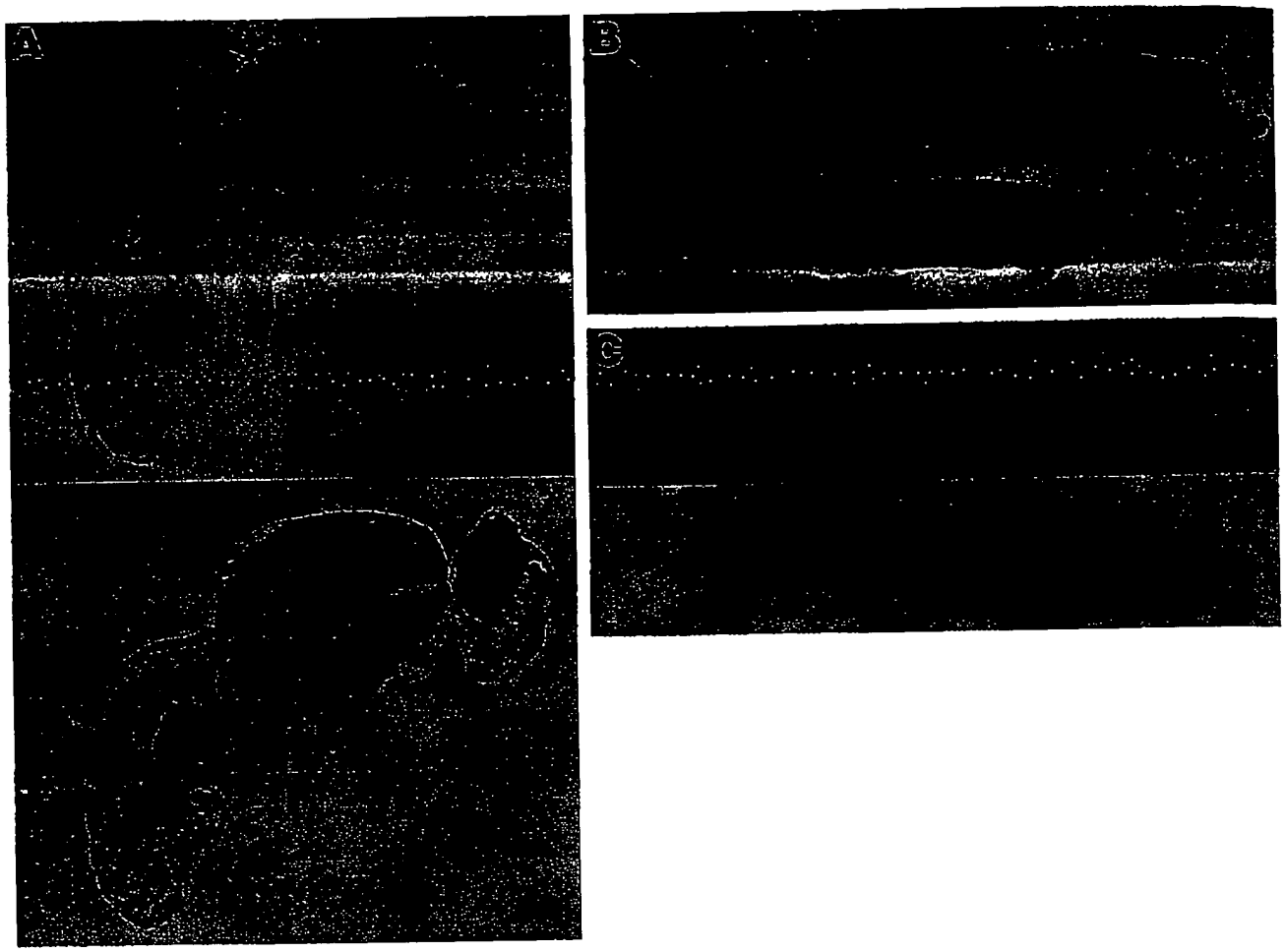

Fig. 2A–C Dox-induced transgene expression detected by an in situ β-gal activity assay. **A** rtTA transgenic line rtTA(2)C1 was crossed to reporter line 7TAdh(2)A2. Young adult progeny were fed with either control sucrose solution (*upper panel*) or sucrose solution containing 1.0 mg/ml dox (*lower panel*) for 48 h, and then allowed to recover for 3 days. Flies were sectioned on a cryostat, and stained for β-gal activity using the chromogenic substrate X-gal. In the control (*upper panel*), low-level β-gal activity is detected primarily in gut tissues. The gut staining indicates some leakiness of expression in the absence of dox, as in non-transgenic *Drosophila* only very faint gut staining is detectable, and only in the abdomen (data not shown, see also Wheeler et al. 1995). In dox-treated (*lower panel*), β-gal activity is detected in all tissues, with the exception of the central region of the indirect flight muscles. All of the indirect flight muscle tissue stains intensely if the staining reaction is allowed to continue for a longer period (data not shown). However with longer staining times the increased intensity of stain in the other body segments obscures the detail of specific tissues, and therefore the results for the shorter staining time are presented. **B** Progeny from the cross rtTA(2)C1 × 7T40(3)B1 were cultured on standard *Drosophila* culture media (upper larva) or *Drosophila* media containing 0.25 mg/ml dox (lower larva). Whole third-instar larvae were stained in situ for β-gal activity. No β-gal activity was detected in the control tissue larvae (*upper larvae*), or in non-transgenic larvae (data not shown). General β-gal activity was detected in the dox treated larvae (*lower larvae*). **C** Repeat of the experiment in **B**, using progeny of the cross rtTA(2)C1 × 7TAdh(2)A2. β-Gal expression in larvae with this reporter was reproducibly less efficient than in the experiment shown in **B**

control food. As seen in Fig. 2B, C, staining of whole third-instar larvae revealed high-level, tissue general induction of β-gal activity with reporter 7T40(3)B1, and somewhat lower level, tissue general induction with reporter 7TAdh(2)A2. The dox-fed larvae were also observed to be slightly smaller than the controls, which may be due to a toxic effect of the dox and/or β-gal expression during development.

Characterization of the response

The induction of β-gal expression can be quantitated by spectrophotometric assay of β-gal activity in fly extracts. This assay was used to optimize the time course of dox treatment. Transactivator line rtTA(2)C1 was crossed to reporter line 7TAdh(3)D1, and the double transgenic progeny were treated with 1.0 mg/ml of dox for 24 h, 48 h, and 48 h plus varying times of recovery without dox. As seen in Fig. 3A, 48 h of treatment plus 3 days of recovery gave the optimal degree of induction (~10-fold). With greater times of recovery, β-gal activity decreased, indicating that the induction is reversible upon withdrawal of dox. The same result was obtained using a different reporter stock, containing the 7T40

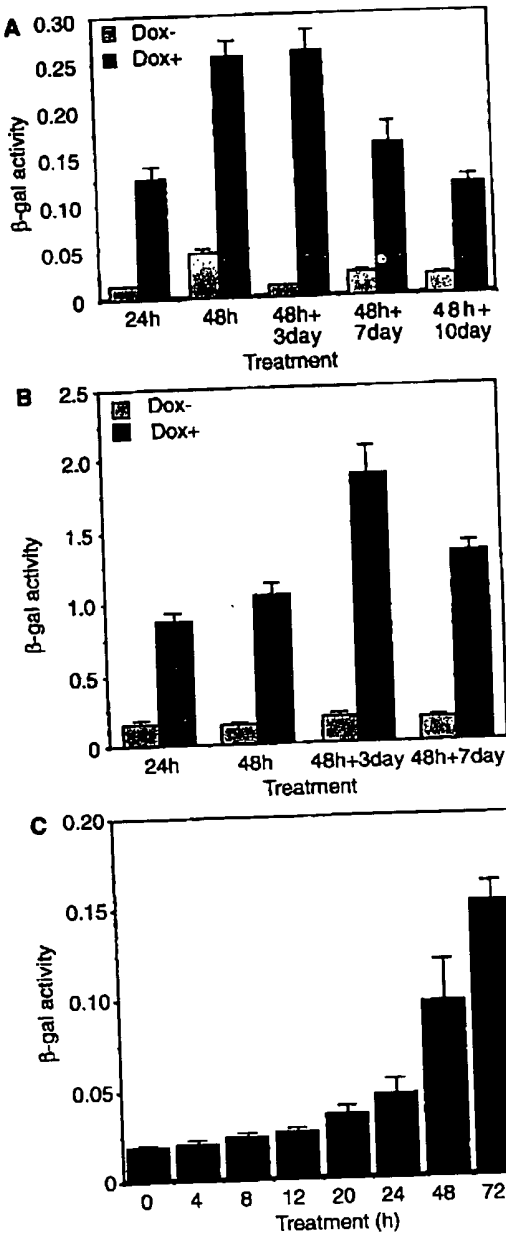

Fig. 3A–C Time course of transgene induction by dox. **A** Young adult progeny of the cross rtTA(2)C1 × 7TAdh(3)D1 were mock treated (Dox-; *stippled bars*), or treated with 1.0 mg/ml dox (Dox+; *black bars*) for the indicated time periods, and allowed to recover as indicated. Triplicate samples containing three flies each were homogenized, and β-gal activity was quantitated using the spectrophotometric assay. β-Gal activity is expressed in relative units, and the averages +/- SD are presented. **B** The experiment in **A** was repeated with a different reporter stock, using young adult progeny from cross rtTA(2)C1 × 7T40(2)E1. **C** The experiment in **A** was repeated using progeny from cross rtTA(2)C1 × 7TAdh(2)A2, and the timecourse for induction was analyzed in greater detail

construct, 7T40(2)E1 (Fig. 3B). Thus, both the *hsp70* core promoter and the *Adh* core promoter can respond to activation by the tetO sequences and the rtTA transactivator.

In transgenic mice the activation by the rtTA transactivator can be quite rapid, with activation by several orders of magnitude occurring in the first 4 h, and maximum levels of activation being achieved by 24 h (Kistner et al. 1996). The timecourse of activation in *Drosophila* was analyzed in greater detail (Fig. 3 C), and found to be significantly slower. In the progeny of the cross rtTA(2)C1 × 7TAdh(2)A2, induction of β-gal by dox feeding was quantitated at intervals between 4 and 72 h. Significant activation was not detected until 8–20 h, and maximal induction required ≥72 h. Similar results were obtained with construct 7TAUG (data not shown). Note that while the level of induction and timecourse was similar for the different reporters in Fig. 3, they are not identical. This probably reflects small differences in the activities of the different reporter insertions, as well as the variability inherent in working with live adult *Drosophila* and administration of dox by feeding.

The *Drosophila* tet-on system was next characterized for the dose response to dox (Fig. 4). Double transgenic adults (rtTA(2)C1 × 7TAdh(2)A2) were fed dox for 48 h and allowed to recover for 3 days (Fig. 4, open circles), or for 96 h plus a 3-day recovery period (closed circles). For 48-h treatment times, β-gal activity was found to increase in response to dox concentrations from 0.01 to 2 mg/ml. Use of the longer 96-h treatment time allowed equivalent levels of β-gal expression with one-tenth as much dox. Thus, longer treatment times reduce the amount of dox required for efficient induction.

To compare the relative activities of the three different reporter constructs, two independent transgenic lines for each reporter were crossed to the rtTA(2)C1 transactivator line (Fig. 5A). Dox-induced β-gal expression

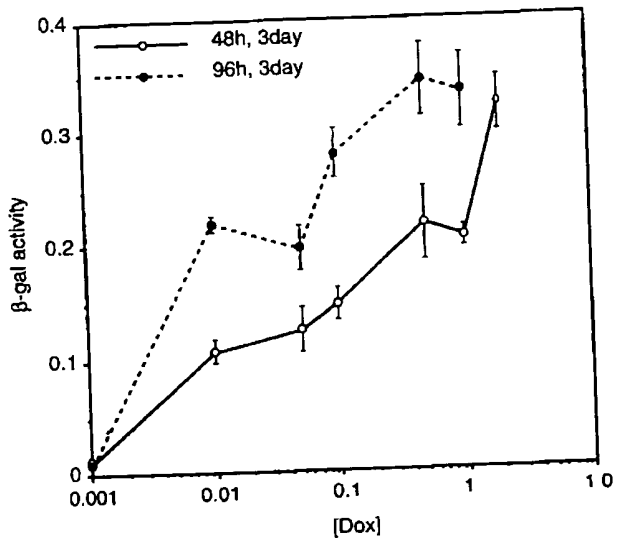

Fig. 4 Dose response of transgene induction by dox. Young adult progeny of cross rtTA(2)C1 × 7TAdh(2)A2 were fed the indicated concentrations of dox for 48 h and allowed to recover for 3 days (*open circles*), or for 96 h plus a 3-day recovery period (*closed circles*). β-Gal expression was quantitated as in Fig. 3

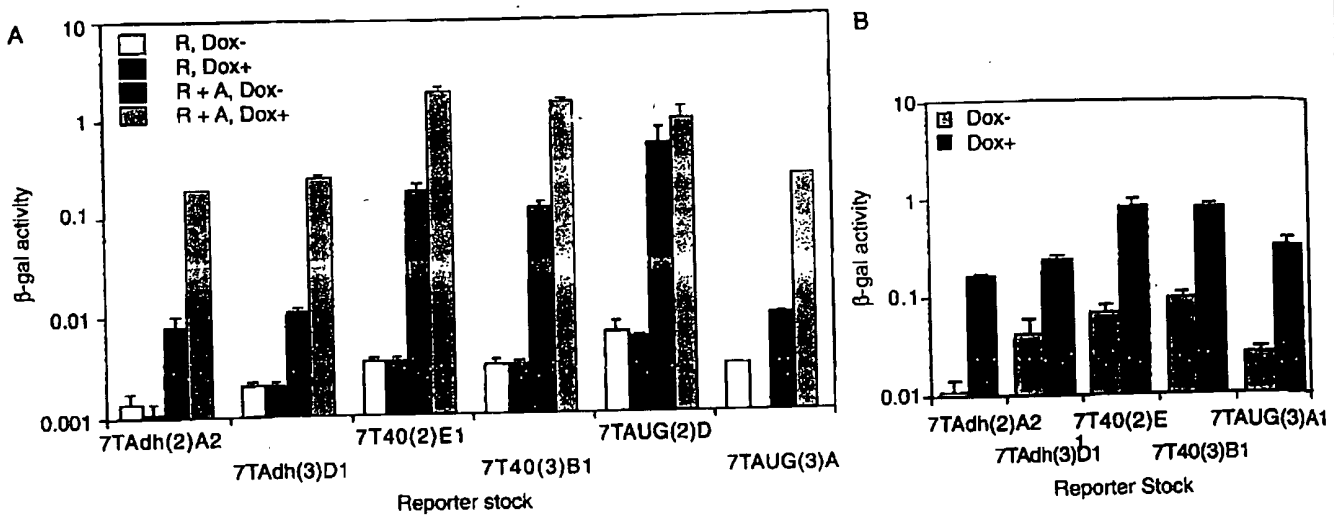

Fig. 5A, B Comparison of transgenic reporter constructs and lines. All dox treatments were for 48 h plus 3 days recovery. **A** Assay in young adults. The indicated reporter lines (R, reporter alone) were assayed with and without dox treatment, as indicated. Each indicated reporter line was also crossed to the rtTA line rtTA(2)C1, and the progeny (R+A, reporter plus activator) were assayed with and without dox treatment, as indicated. **B** Assay in old adults. rtTA line rtTA(2)C1 was crossed to each indicated reporter line, and old adult progeny were assayed with and without dox treatment, as indicated. β-Gal expression was quantitated as in Fig. 3

was observed with all three constructs, with induction factors ranging from 12- to 25- fold. In general, the 7T40 reporter construct gave higher levels of β-gal expression than the other two reporter constructs; however, the background expression in the absence of dox was also higher. Thus, the induction factor achieved was similar for each of the three reporter constructs.

To determine if the system functions during aging of *Drosophila*, the activity of each reporter construct was also assayed in senescent (30-day-old) flies (Fig 5B). Each reporter was found to support dox-induced β-gal expression in senescent flies, with induction factors ranging from 8- to 15-fold.

The dox-inducible system is dependent upon efficient, general expression of the transactivator construct, rtTA. Because the chromosomal site of insertion of the rtTA transgene can affect the level of expression, different independent rtTA transgenic lines may vary in their activity. To compare their activities, each of 13 independent rtTA transgenic lines was crossed to the 7TAdh(3)D1 reporter, and the efficiency of induction of β-gal by dox was quantitated (Fig. 6A). The different independent rtTA lines were found to vary in activity, both with regard to the amount of background β-gal activity in the absence of dox, and with regard to the maximum level of induction in the presence of dox. Transgenic transactivator line rtTA(3)E2 appeared to be the best: in the absence of dox, background β-gal levels were as low as in flies carrying the reporter construct in the absence of any transactivator, and dox treatment yielded a 40-fold induction. To confirm this result, each

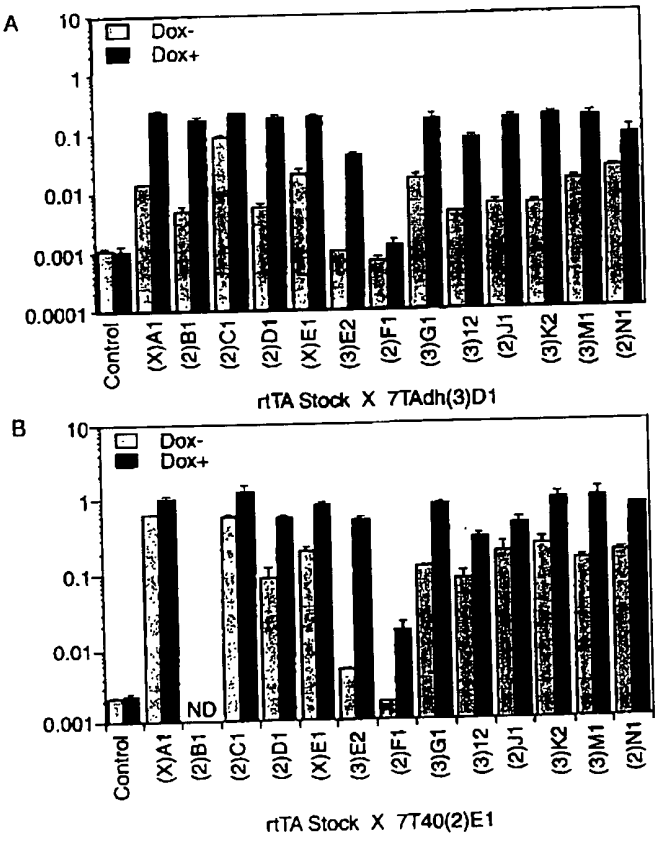

Fig. 6A, B Comparison of different transgenic transactivator (rtTA) lines. All dox treatments were for 48 h plus 3 days recovery. **A** The indicated rtTA lines were each crossed to reporter line 7TAdh(3)D1. The young adult progeny from each cross were assayed without dox treatment (Dox-; *stippled bars*), and with 1.0 mg/ml dox treatment (Dox+; *black bars*), as indicated. Control was the reporter line 7TAdh(3)D1 alone. **B** The experiment in **A** was repeated using the reporter line 7T40(2)E1. ND, not done

transactivator stock was also tested in combination with reporter stock 7T40(2)E1 (Fig. 6B). Again the various transactivator lines varied with regard to background and maximal level of induction, and their activity relative to each other was similar to that observed using the 7TAdh reporter. Line rtTA(3)E2 was again found to have the lowest background, and to be the most active, yielding 100-fold induction of β-gal in response to dox.

One potential use of the dox-inducible system in *Drosophila* is in the analysis of the effects of specific genes on the aging process. Ideally, for such experiments, the system itself should not have any effect on life span. To characterize the system for effects on life span, several genetic backgrounds were tested for longevity, with and without dox feeding. Wild-type flies exhibited no negative effects on life span when fed with 0.1 mg/ml dox (Fig. 7A). Double transgenic flies (rtTA(2)C1 × 7TAdh(2)A2) expressed high levels of β-gal in response to 0.1 mg/ml dox (Fig. 4), and this expression also had no detectable negative effects on lifespan (Fig. 7A). Finally, a different combination of transactivator and reporter were tested. Transactivator rtTA(X)A1 was crossed to reporter 7TAdh(3)D1 and age-synchronized cohorts of adult flies were treated throughout their adult lifespan with no dox, 0.01 mg/ml dox, or 1.0 mg/ml dox (Fig. 7B). Treatment with 1.0 mg/ml dox was found to have a negative affect on life span. However, treatment with 0.01 mg/ml dox had no detectable affect on life span. The same results were obtained with several other combinations of rtTA and reporter lines (data not shown). Since 0.01 mg/ml dox and 0.1 mg/ml dox allow high-level induction of β-gal (Fig. 4), and have no detectable effect on lifespan (Fig. 7), these results suggest that the system should be useful for assaying the effects on life span of overexpression of specific genes.

Discussion

The hybrid transcriptional activator (rtTA) consisting of the rtR fused to the transcriptional activation domain of the herpes virus protein VP16 has previously been shown to be capable of supporting dox-induced transcription in transgenic mice. The experiments presented

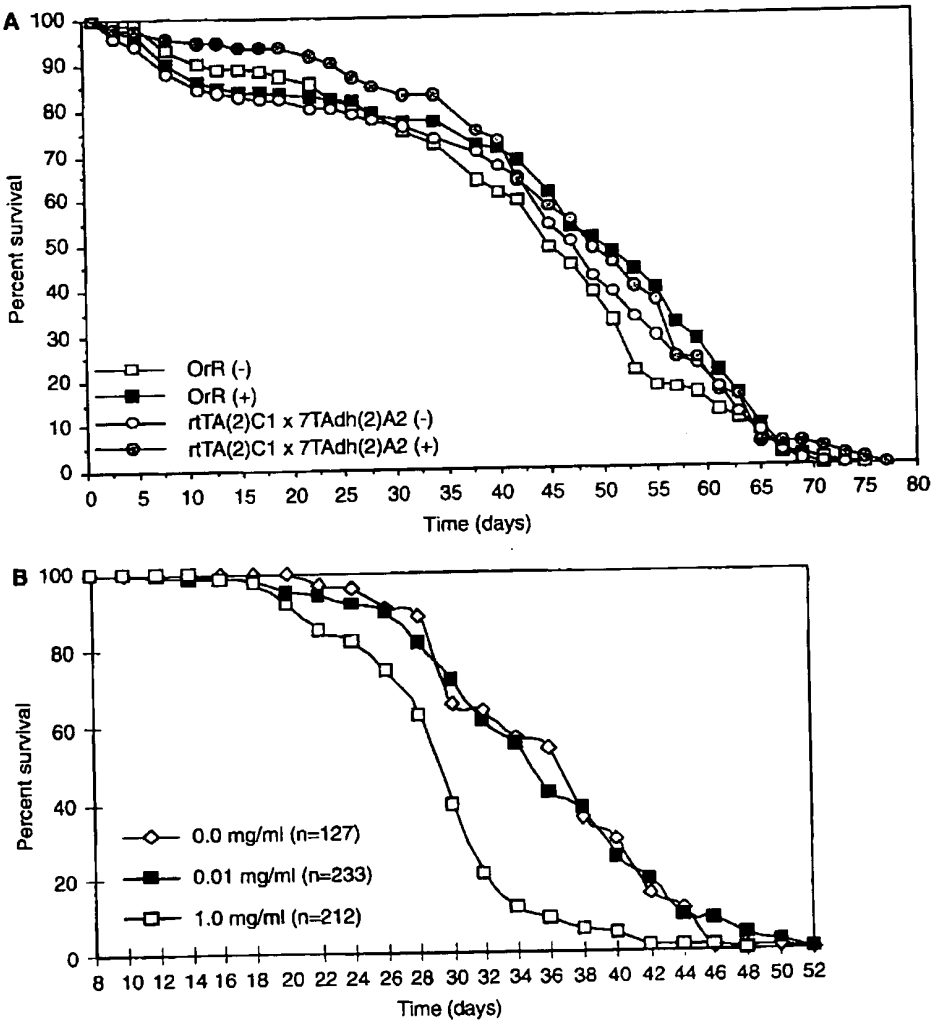

Fig. 7A, B Affect of dox-induced β-gal expression on *Drosophila* adult life span. **A** Wild-type Oregon R strain flies were treated throughout their adult lifespan either without dox (*open squares*) or with 0.1 mg/ml dox (*filled squares*), at 25° C. Flies were fed or mock-fed dox for 2 days, and then allowed to recover on standard media for 2 days, and this regimen was repeated until all the flies had died. The percentage of flies surviving is plotted as a function of time in days. The same experiment was performed using progeny from cross rtTA(2)C1 × 7TAdh(2)A2 grown in the absence of dox (*open circles*) or in the presence of 0.1 mg/ml dox (*closed circles*). At least 200 flies were used for each of the four survival curves. **B** Progeny from the cross rtTA(X)A1 × 7 TAdh(3)D1 were grown throughout their adult life span in the absence of dox (*open diamonds*), or in the presence of 0.01 mg/ml dox (*filled squares*), or 1.0 mg/ml dox (*open squares*), at 25° C. The number of flies used (n) for each survival curve is indicated. The different genotypes assayed in **A** and **B** vary in life span relative to each other, which is not unexpected due to the large effects of genetic background on life span (Curtsinger et al. 1995; Tower 1996)

here demonstrate that this rtTA functions in transgenic *Drosophila*, and can activate transcription at both the *hsp70* and *Adh* core promoters when they are linked to tetO sequences. Since all three reporter constructs performed similarly, the results suggest that in this system there is no significant difference in the effectiveness of the SV40 poly(A) signal relative to the *hsp70* poly(A) signal, and no significant difference between the effectivness of the *Adh*, *hsp70* and *Ubx* 5′ UTR regions. Dox-induced transgene expression was detected in all tissues, and induction ranged from 10- to 100-fold. Different transgenic lines containing the rtTA construct varied considerably in activity. Variation was observed in the maximal level of induction achieved, and in the amount of background activity observed in the absence of dox. This variation is likely to be due to chromosomal position effects on the expression of the rtTA transposon, and perhaps to other differences in the genetic background of the lines. The different transgenic lines of the reporter constructs also varied in activity, most probably for the same reasons. The maximum degree of induction that was achieved was 100-fold. This is dramatically less than the five orders of magnitude induction obtained with the tet-on system in transgenic mice (Kistner et al. 1996). The maximal induction achieved in *Drosophila* is limited by at least two factors: First, the reporter constructs are slightly leaky, in that variable, low-level β-gal activity is detected even in the absence of the rtTA transactivator. Second, the rtTA transactivator appears to be partially active even the absence of dox treatment, in that reporter plus transactivator was often more active than reporter alone. The first problem might be addressed by protecting the reporter constructs from position effects with insulator elements (Roseman et al. 1993), and/or by identifying a less leaky core promoter. The second problem can be mitigated by identifying particular rtTA lines, such as rtTA(3)E2, which exhibit less background activation. Finally, we hypothesize that the herpes virus VP16 transcriptional activation domain used to create the rtTA transactivator may be better suited to interaction with the mammalian transcriptional machinery than with the *Drosophila* transcriptional machinery. This possibility may also be relevant to the slower time course of induction observed in *Drosophila*.

Despite its limitations, this inducible system has several potential advantages relative to the use of heat shock gene promoters. The dox-inducible system should be useful for studying hsps, as it will allow the investigator to induce the expression of a single hsp, and potentially inhibit its expression with antisense RNA, without inducing the endogenous heat shock response. The dox- inducible system also allows the investigator to induce a gene of interest at any time during the life cycle. This is particularly relevant to study of the aging process (Curtsinger et al. 1995; Tower 1996), where it is often desirable to alter gene expression specifically in the adult. For example, constitutive over-expression of Cu/Zn SOD may have beneficial effects on *Drosophila* life span but it also appears to have toxic affects during pupal development (Reveillaud et al. 1991). Using the dox-inducible system it should be possible to avoid toxic effects during development and cause over-expression of transgenes specifically in the adult where beneficial effects on life span may be more apparent.

The dox-inducible system should be readily adaptable to tissue-specific induction. Replacement of the constitutive *Actin5C* promoter in the transactivator construct rtTA with a tissue-specific promoter should provide tissue-specific expression of the rtTA transactivator and thus tissue-specific induction of the reporter. An elegant system for tissue-specific expression of the yeast GAL4 transactivator has been developed for *Drosophila* (Brand and Perrimon 1993; Brand and Dormand 1995). In this case tissue-specific expression of the GAL4 transactivator is driven by an "enhancer-trap" system: the transactivator is under the control of a weak transcriptional promoter which can become activated in a tissue- and temporal-specific manner when the P element inserts near transcriptional enhancer sequences in the chromosome. The large variety of tissue- and temporal-specific GAL4 transactivator expression patterns generated thus allows tissue- and temporal-specific expression of "reporter" type constructs containing GAL4 binding sites in their promoters. This system could be adapted to drive expression of the rtTA transactivator, thus creating a large variety of tissue-specific expression patterns inducible by dox.

Finally, it may be possible to create dox-dependent mutations in *Drosophila*. P element constructs with transcriptional promoters directed out of the end of the P element can cause over-expression and/or mis-expression of genes near the site of insertion, sometimes causing dominant mutations (Rorth 1996; Hay et al. 1997). Creation of a P element with a dox-inducible promoter directed out of the P element into flanking DNA sequences should sometimes cause dox-induced over-expression of a gene near the insertion site. This method should thus yield conditional (dox-dependent), dominant, gain-of-function mutations which would be useful for many types of genetic analyses; such experiments are now underway.

Acknowledgements We thank Hermann Bujard for providing constructs. J.C.W. was supported by a pre-doctoral training grant from the National Institute on Aging (AG00093). This work was supported by a grant from the Department of Health and Human Services, National Institute on Aging to J.T. (AG11644)

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APPENDIX H

GENETIC ENGINEERING OF MAMMALIAN EMBRYOS[1,2,3]

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University of Pennsylvania, Philadelphia 19104

ABSTRACT

A gene consisting of the mouse metallothionein I promoter/regulator (MT) fused to the human growth hormone (hGH) structural gene (MThGH) was microinjected into rabbit, sheep and pig eggs. Visualization of nuclear structures was accomplished by interference-contrast (I-C) microscopy for rabbit and sheep eggs and by centrifugation and I-C microscopy for pig eggs. The gene integrated into the chromosomes of each species with an efficiency of 13% in rabbits, 1% in sheep and 10% in pigs. Human GH mRNA was detected in the liver of transgenic rabbits as well as tail and ear samples of pigs. Immunoreactive hGH was present in the serum of a transgenic rabbit and plasma of most transgenic pigs. In several pigs hGH levels increased between birth and 90 d of age. The presence of substantial quantities of hGH in plasma of pigs did not increase postnatal somatic growth rates. Founder animals will be bred and their transgenic and control progeny used to assess the effects of hGH on feed efficiency and carcass composition. These experiments demonstrate the feasibility of introducing foreign genes into the genome of several animal species by microinjection of eggs.

(Key Words: Rabbits, Sheep, Pigs, Somatotropin, Genetic Engineering, Genes.)

Introduction

A variety of genes, particularly those involved in regulation of growth, have been introduced into mice. The rat and human growth hormone (GH) structural genes become stably integrated into the mouse genome, but they are not efficiently expressed (Hammer et al., 1984a). In contrast, transgenic mice harboring fusion genes consisting of the mouse metallothionein promoter/regulator (MT) fused to the rat (r), bovine (b) or human (h) GH structural gene exhibit high, metal inducible levels of GH mRNA in many organs, particularly the liver and intestine (Palmiter et al., 1982, 1983; Hammer et al., 1985a). Most of the mice have substantial levels of foreign GH in their sera and show markedly enhanced growth rates and a doubling in adult body size. The genes generally become stably integrated into the germline and are heritable with the transgenic offspring exhibiting enhanced growth.

The application of gene transfer techniques to domestic species has been impeded primarily by the inability to visualize egg nuclei. By using interference-contrast microscopy (I-C) for rabbit and sheep eggs (Hammer et al., 1985c), and centrifugation and I-C microscopy for pig and cow eggs (Wall et al., 1985), nuclei become visible and can be microinjected. The purpose of this study was to determine if microinjected genes could become incorporated into the genome of other mammalian species and to determine if hGH gene expression would enhance growth rates. To test such possibilities, we microinjected mouse metallothionein-human growth hormone (MThGH) fusion gene into the nuclei of rabbit, sheep and pig eggs and examined the resulting animals for gene integration and expression.

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[3] Presented by the senior author at a symposium on "Future Impact of Biotechnology in Animal Science," held August 14, 1985 at the 77th Annu. Meet. of the Amer. Soc. of Anim. Sci., Univ. of Georgia, Athens.

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TABLE 1. SUPEROVULATION IN SHEEP AND PIGS

Group	Sheep	Pig
No. of females injected	205	218
No. of females that ovulated	196	182
No. of eggs collected/female that ovulated	10.4 ± .5[a]	23.4 ± 2.6
Egg recovery after expected time of ovulation, h[b]	18-25	18-27

[a]Mean ± SE.

[b]Ewes were expected to ovulate 50 to 55 h following sponge removal; gilts were expected to ovulate 40 to 44 h after administration of human chorionic gonadotropin (Hunter and Dziuk, 1968; Hunter, 1972).

Materials and Methods

Animal Treatment. The methods of hormone administration, estrous synchronization, breeding, embryo collection and transfer for mice, rabbits, sheep and pigs have been previously reported (Hammer et al., 1985c).

Superovulation Efficiency. Progestogen-impregnated intravaginal sponges were used in combination with multiple injections of porcine follicle stimulating hormone (FSH) to obtain superovulation in ewes (Armstrong and Evans, 1983, Armstrong et al., 1983). With this regimen we obtained both a high mean ovulation rate (16.9%) and a high mean embryo yield (table 1). The relatively low embryo recovery rate (64%) was probably caused by fimbrial adhesions and scarring of oviductal tissue due to multiple use of some donors and failure to recover any eggs in some ewes that ovulated while the progesterone pessary was in place.

In pigs, standardized superovulation techniques were utilized (Wall et al., 1985). Egg yield per ovulating female was high (table 1), but 11.5% of female failed to ovulate and 11% developed cystic ovaries, some of which ovulated. The number of ovulation points per gilt could not be determined accurately because of ovarian adhesions and scarring caused by multiple uses of some donors.

Microinjection and Nucleic Acid Analysis. A few hundred copies of a 2.6-kilobase BstEII/EcoRI linear DNA fragment isolated from a plasmid containing the MThGH gene was microinjected as described (Hammer et al., 1985c). The number of copies of the MThGH gene that integrated was estimated by quantitative dot hybridization. The DNA from some of the transgenic animals also was analyzed by Southern blotting. The MThGH mRNA was measured by solution hybridization utilizing a complementary end-labeled 21-base oligonucleotide probe (Ornitz et al., 1985).

Human Growth Hormone Analysis. Plasma samples were analyzed in duplicate at 2.5 and 10 μl by radioimmunoassay using a hGH antibody (NIAMDD-anti-hGH-1) and reference standard (NIAMDD-hGH-RP-1) provided by Dr. Raiti[7] (National Hormone and Pituitary Program) and radioiodinated (Miller et al., 1985) hGH (LER-hGH-1) provided by Dr. L. Reichert[8]. The hGH assay did not cross-react with porcine GH. Cross-reactivity with rabbit GH was not determined but plasma from non-transgenic rabbits did not cause displacement in the assay. Plasma samples were designated as negative for hGH when an hGH concentration was less than 2 ng/ml (approximately 15% inhibition of binding from buffer controls with 10-μl samples).

Results and Discussion

Visualization of Egg Nuclei. Visualization of nuclei in eggs of domestic livestock has been the greatest impediment to the successful application of gene transfer by microinjection. Nuclei of mouse and rabbit eggs (figure 1a) are easily discernible with conventional light microscopic techniques. However, the cytoplasm of sheep eggs and, particularly, pig and cow eggs is so optically dense that other methods for visualization had to be developed. Deoxyribonucleic acid-specific fluorochromes have recently been utilized to localize nuclei in cow

[7]Baltimore, MD.

[8]Albany Med. College, Albany, NY.

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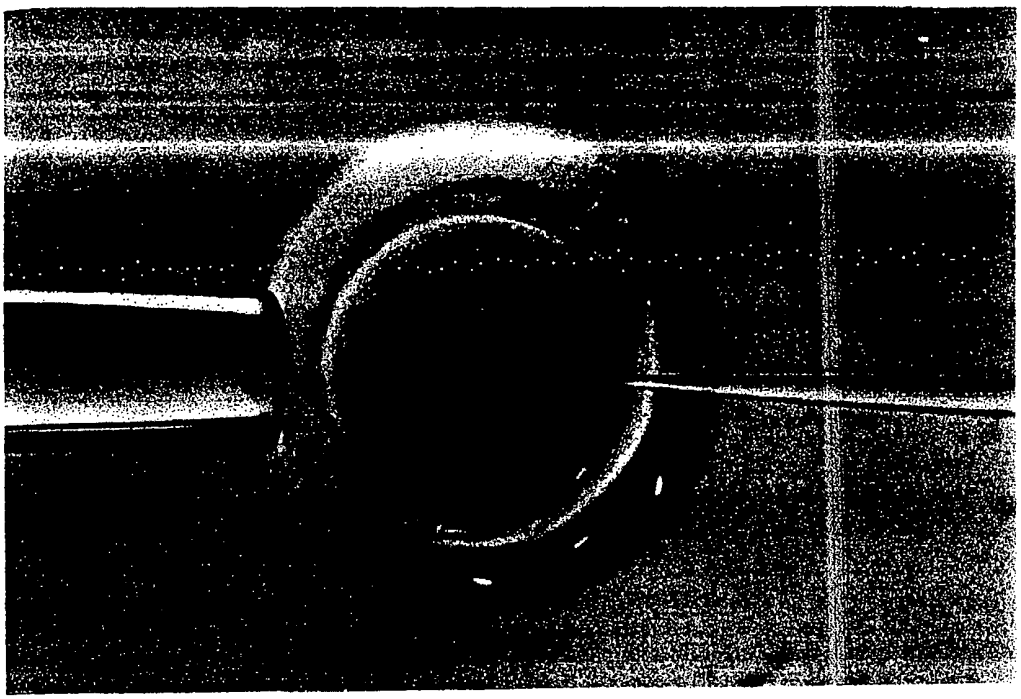

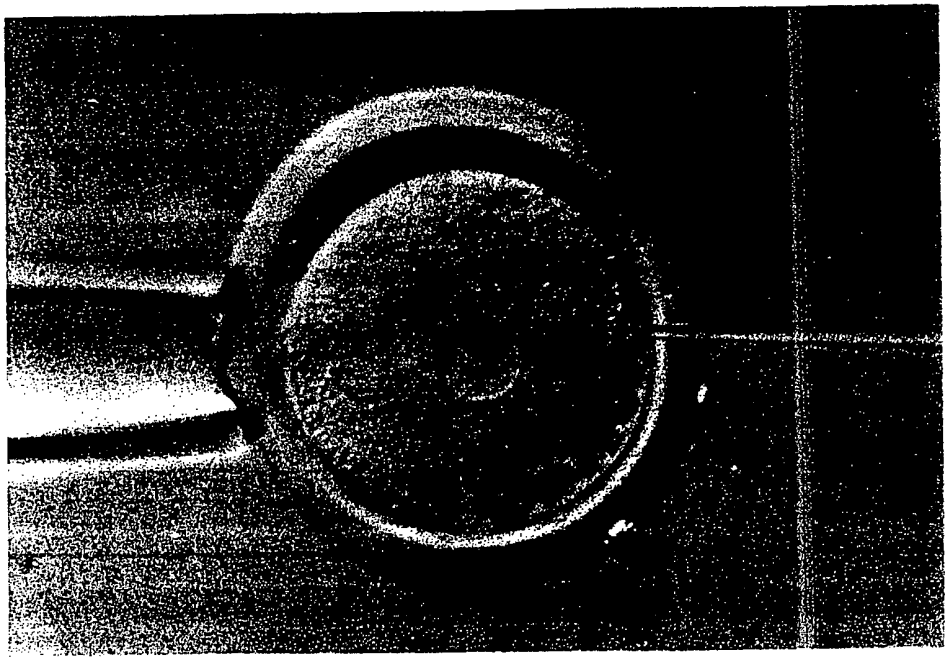

Figure 1. Interference-contrast photomicrographs of one-cell fertilized ova from rabbit (a) and sheep (b) following microinjection. Ova are held by a blunt holding pipette (diameter approximately 50 μm), and an injecting pipette (tip diameter approximately 1.5 μm) is seen within a pronucleus immediately following injection of buffer containing DNA. Microinjection is carried out under 250 × magnification using a Leitz microinjection apparatus.

(Crister et al., 1983; Minhas et al., 1984), pig (Pursel et al., 1985) and sheep eggs (R. E. Hammer, C. E. Rexroad and R. L. Brinster, unpublished observations). However, the applications of these techniques as an aid to microinjection seems limited because short exposure of Hoechst 33342- and 33258-stained pig (Wall et al., 1985) and sheep eggs to ultraviolet light (100 W) was embryotoxic.

We found that sheep egg nuclei can be located with the aid of I-C microscopy (figure 1b). Examination of fertilized sheep eggs by I-C microscopy and fluorescent microscopy using a DNA-specific fluorochome indicated that at least 80% of fluorescent pronuclei were visible with I-C microscopy. Pig and cow ova are so optically opaque that neither phase, dark field nor I-C microscopy (figure 2a) are useful for visualizing nuclei. Centrifugation has been used to stratify the cytoplasm of sea urchin embryos into a number of distinct layers, one of which contains the nucleus and is devoid of lipid and pigment (Harvey, 1956). Therefore, we explored the use of centrifugation to aid in visualizing nuclei in pig, cow and sheep eggs (Wall et al., 1985). Centrifugation of pig (figure 2b) and cow (figure 3) ova at 15,000 × g for 5 to 10 min markedly stratifies the cytoplasm, leaving the pronuclei or nuclei accessible to microinjection. While the cytoplasm of sheep eggs stratifies when subjected to 15,000 × g, centrifugation does not greatly improve visualization.

Microinjection and Embryo Survival. To determine the effects of 12 h of culture and the toxicity of nuclear-injected DNA (2 to 5 pl of 2 ng/μl) on sheep and pig egg development, we transferred cultured control and microinjected eggs into recipients and examined the development of recovered eggs.

Only 26% of uninjected one- and two-cell sheep eggs and 52% of centrifuged uninjected pig eggs survived to blastocysts (table 2). Such a low percentage of development may be due, at least in part, to the trauma of extended culture. Injection of DNA further reduced embryo development by approximately 50% in each species (table 2).

As indicated by a decrease in the number of eggs that developed to the morula/blastocyst stage in culture, and the lower recovery of injected eggs as fetuses from pseudopregnant females, microinjection of buffer into the male pronucleus of one-cell eggs was detrimental to development. Injection of a DNA dose (2 ng/μl) that provides a high integration efficiency (27%) further diminishes mouse egg survival to blastocysts and fetuses (Brinster et al., 1985).

Integration Efficiency. In mice, both the form and concentration of DNA, as well as the site of gene injection, are important factors for efficient integration (Brinster et al., 1985). Linear molecules of DNA integrate with much higher efficiency than circular forms, and nuclear deposition of DNA is more effective than cytoplasmic deposition. Because of our initial difficulty in visualizing pig egg nuclei we piloted gene transfer in pigs by cytoplasmic injection of one- and two-cell eggs. None of the fetuses that developed from these eggs contained integrated MThGH sequences (table 3). In contrast, nuclear injection of centrifuged pig eggs resulted in an integration efficiency of approximately 10%. Integration frequency was 12.8 and 1.3% in the rabbit and sheep, respectively (table 3).

These efficiencies of integration, particularly in the sheep, are much lower than rates we currently obtain in mice (27%). Because of the difficulty and expense in obtaining large numbers of pig and sheep eggs, it has been difficult to examine factors which may influence integration rate. The frequency of integration for these species (table 3) has resulted from using conditions that were shown to be optimal for mouse eggs (Brinster et al., 1985). Both the age of the ovum and the structure of the host chromosomes may be important factors for integration. Efficiency in the rabbit has been improved to approximately 30% by injecting eggs in the early pronuclear stage (data not shown), as compared with injecting near the time of one-cell cleavage. Such considerations may be equally important in other species.

Reasons for the extremely low integration efficiency in sheep is puzzling. Although none of the lambs that developed from cytoplasmically injected eggs contained integrated DNA (data not shown), we anticipated markedly improved efficiency when we began microinjecting into the nucleus. Such a dramatic increase has not occurred.

The pattern of DNA integration in MThGH rabbits and pigs was similar to that observed in mice. The number of copies of the integrated gene varied from 1 to 490 per cell. Southern analysis verified that all the transgenic rabbits and pigs contained intact copies of MThGH,

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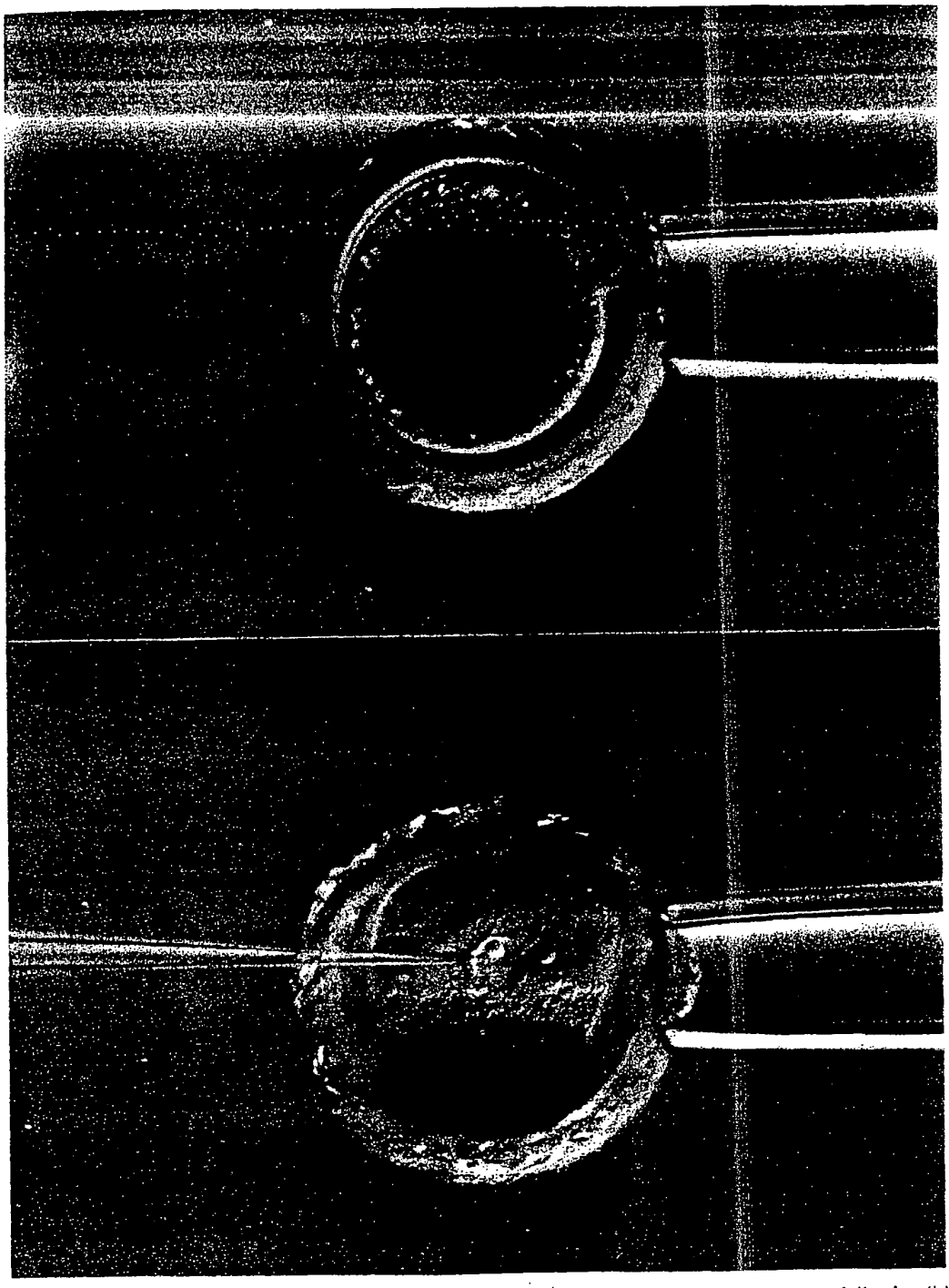

Figure 2. Interference-contrast photomicrographs of one-cell fertilized pig ova before (a) and following (b) centrifugation and microinjection. Porcine ova have been centrifuged at 15,000 X g for 3 min to reveal the pronuclei.

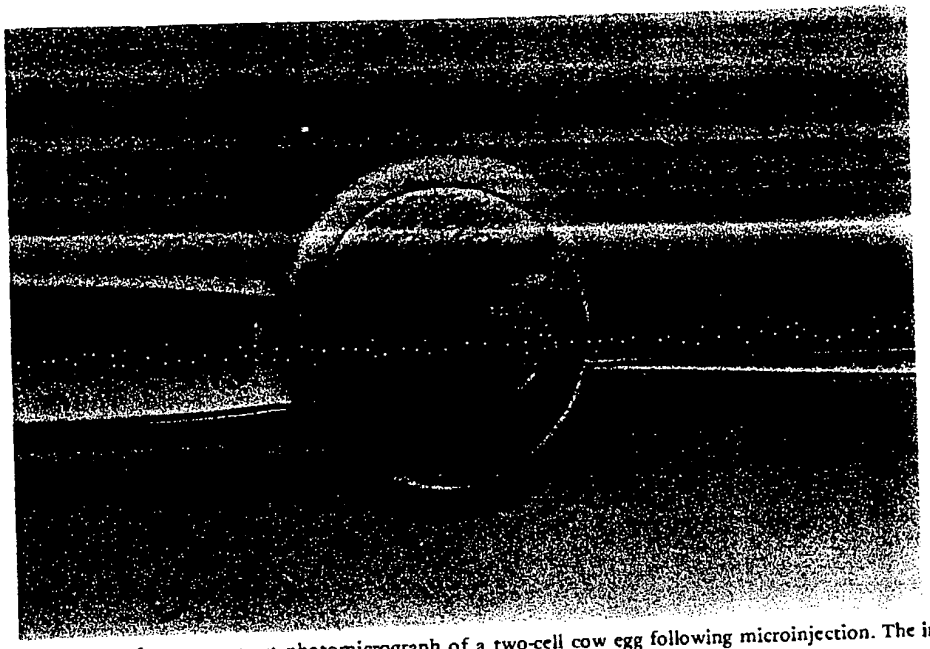

Figure 3. Interference-contrast photomicrograph of a two-cell cow egg following microinjection. The injecting pipette is seen within the nucleus of a blastomere following injection.

with many oriented in tandem head-to-tail arrays, as well as some in head-to-head configuration. Restriction analysis of DNA isolated from the one transgenic sheep indicated that the MThGH gene was not intact.

Expression of MThGH Genes. The mouse metallothionein gene is expressed in many tissues, particularly in large secretory organs such as liver, kidney and intestine. The gene has a transcriptional promoter that is regulated by heavy metals such as cadmium and zinc. The promoter functions when transfected into human (Mayo et al., 1982) and rabbit (R. D. Palmiter, unpublished observations) tissue culture cells, and has been shown to provide regulatable expression of the rat, bovine and human GH genes in transgenic mice. These results led us to choose this promoter to begin our work in introducing genes into other mammalian species.

Four of the 16 MThGH rabbits had detectable levels of hGH mRNA in the liver and one animal (200-3) had substantial levels (table 4). Tissue-specific expression was examined in more detail in one of the founder rabbits (163-3). The small intestine contained the

TABLE 2. EFFECT OF DNA MICROINJECTION ON EMBRYO SURVIVAL TO BLASTOCYST

Group	Sheep	Pig
Uninjected[a]	28/108 (26)[c]	22/42 (52)
Microinjected[ab] with DNA	24/233 (10)	39/165 (23)

[a]Only eggs with visible pronuclei or nuclei were used. All pig eggs were centrifuged to permit visualization of nuclei. Both uninjected and injected eggs were in culture tubes and(or) microdrops for approximately 12 h before transfer into recipient ewes or gilts. Eggs were recovered from sheep at 8 d and pigs at 6 d following transfer. Recovered eggs having a visible cavity were classified as blastocysts.

[b]Pronuclei or nuclei of sheep and pig eggs were microinjected with a solution containing 2 ng/μl of MThGH genes. Eggs were transferred approximately 3 to 5 h following microinjection of DNA.

[c]Blastocysts per eggs transferred. Numbers in parentheses represent percent.

highest quantity of hGH mRNA, with the liver, kidney and testis containing lower but detectable levels (data not shown). In pigs, the hGH mRNA content of tail or ear samples was quantitated to avoid any adverse consequences resulting from partial hepatectomy. Nevertheless, MThGH mRNA was detectable in many of the founder pigs (table 5). A more detailed examination of tissue expression in pigs will be undertaken once founder animals have been bred and germ-line transmission has been verified.

To determine if MThGH expression resulted in production of immunoassayable hGH, we quantitated hGH levels in the serum of a transgenic rabbit and the plasma of transgenic pigs. A rabbit had a substantial level of serum hGH. Eleven of the 18 founder pigs had detect-

TABLE 3. EFFICIENCY OF GENE TRANSFER INTO ANIMALS

Species	Site of injection	Eggs[a] injected and transferred	Fetuses and neonates[b]	Animals with integrated gene[c]	Animals expressing the gene[d]
Mice[e]	Cytoplasm	890	224 (25)	2 (1)	ND
	Pronucleus	946	111 (12)	39 (35)	23 (59)
Rabbit	Pronucleus	1,907	218 (11)	28 (13)	4 (25)
Sheep	Pronucleus	1,032	73 (7)	1 (1)	ND
Pig	Cytoplasm	485	42 (9)	0 (0)	
	Pronucleus	2,035	192 (9)	20 (10)[f]	11[g] (61)

[a]Eggs were microinjected with a solution of linear DNA containing MThGH genes.

[b]The number of animals (fetuses, stillborns and neonates) resulting from injected ova. Numbers in parentheses are the number of animals produced/total eggs injected.

[c]The number of animals that incorporated the injected DNA. Numbers in parentheses are the number of MThGH animals/total number of animals produced.

[d]The number of animals containing MThGH mRNA. Numbers in parentheses are the number of MThGH animals expressing the gene/total number of MThGH animals analyzed. Sixteen rabbits were analyzed for mRNA. ND = not determined.

[e]Data for mice are from Brinster et al. (1985).

[f]Integration efficiency for pigs based on combined data from female recipients bearing injected eggs only or injected and control eggs (Hammer et al., 1985c).

[g]Pigs containing detectable plasma human growth hormone. Plasma from 18 animals was analyzed for hGH.

TABLE 4. CHARACTERISTICS OF MThGH TRANSGENIC RABBITS AND SHEEP

Species	Animal[a] and sex	Gene copy, no./cell	hGH mRNA[b], molecules/cell	Immunoassayable hGH, ng/ml
Rabbit	163-3[c]	3	15	250
Rabbit	200-3	8	920	ND
Rabbit	68-3	28	39	ND
Rabbit	221-1	40	140	ND
Sheep	693-1[d]	1	ND[e]	ND

[a]Animals without identified gender were sacrificed as fetuses.

[b]Liver MThGH mRNA was quantitated.

[c]Male.

[d]Female.

[e]ND = not determined.

icroinjection. The inject-

ervations) tissue cul- shown to provide the rat, bovine and nsgenic mice. These is promoter to begin r genes into other

l rabbits had detect- in the liver and one ntial levels (table 4). was examined in he founder rabbits tine contained the

BLASTOCYST

Pig
22/42 (52)
39/165 (23)

permit visualization of pproximately 12 h be- at 6 d following trans-

ing 2 ng/μl of MThGH

Figure 3. Interference-contrast photomicrograph of a two-cell cow egg following microinjection. The injecting pipette is seen within the nucleus of a blastomere following injection.

with many oriented in tandem head-to-tail arrays, as well as some in head-to-head configuration. Restriction analysis of DNA isolated from the one transgenic sheep indicated that the MThGH gene was not intact.

Expression of MThGH Genes. The mouse metallothionein gene is expressed in many tissues, particularly in large secretory organs such as liver, kidney and intestine. The gene has a transcriptional promoter that is regulated by heavy metals such as cadmium and zinc. The promoter functions when transfected into human (Mayo et al., 1982) and rabbit (R. D. Palmiter, unpublished observations) tissue culture cells, and has been shown to provide regulatable expression of the rat, bovine and human GH genes in transgenic mice. These results led us to choose this promoter to begin our work in introducing genes into other mammalian species.

Four of the 16 MThGH rabbits had detectable levels of hGH mRNA in the liver and one animal (200-3) had substantial levels (table 4). Tissue-specific expression was examined in more detail in one of the founder rabbits (163-3). The small intestine contained the

TABLE 2. EFFECT OF DNA MICROINJECTION ON EMBRYO SURVIVAL TO BLASTOCYST

Group	Sheep	Pig
Uninjected[a]	28/108 (26)[c]	22/42 (52)
Microinjected[ab] with DNA	24/233 (10)	39/165 (23)

[a]Only eggs with visible pronuclei or nuclei were used. All pig eggs were centrifuged to permit visualization of nuclei. Both uninjected and injected eggs were in culture tubes and(or) microdrops for approximately 12 h before transfer into recipient ewes or gilts. Eggs were recovered from sheep at 8 d and pigs at 6 d following transfer. Recovered eggs having a visible cavity were classified as blastocysts.

[b]Pronuclei or nuclei of sheep and pig eggs were microinjected with a solution containing 2 ng/μl of MThGH genes. Eggs were transferred approximately 3 to 5 h following microinjection of DNA.

[c]Blastocysts per eggs transferred. Numbers in parentheses represent percent.

highest quantity of hGH mRNA, with the liver, kidney and testis containing lower but detectable levels (data not shown). In pigs, the hGH mRNA content of tail or ear samples was quantitated to avoid any adverse consequences resulting from partial hepatectomy. Nevertheless, MThGH mRNA was detectable in many of the founder pigs (table 5). A more detailed examination of tissue expression in pigs will be undertaken once founder animals have been bred and germ-line transmission has been verified.

To determine if MThGH expression resulted in production of immunoassayable hGH, we quantitated hGH levels in the serum of a transgenic rabbit and the plasma of transgenic pigs. A rabbit had a substantial level of serum hGH. Eleven of the 18 founder pigs had detect-

TABLE 3. EFFICIENCY OF GENE TRANSFER INTO ANIMALS

Species	Site of injection	Eggs[a] injected and transferred	Fetuses and neonates[b]	Animals with integrated gene[c]	Animals expressing the gene[d]
Mice[e]	Cytoplasm	890	224 (25)	2 (1)	ND
	Pronucleus	946	111 (12)	39 (35)	23 (59)
Rabbit	Pronucleus	1,907	218 (11)	28 (13)	4 (25)
Sheep	Pronucleus	1,032	73 (7)	1 (1)	ND
Pig	Cytoplasm	485	42 (9)	0 (0)	
	Pronucleus	2,035	192 (9)	20 (10)[f]	11[g] (61)

[a]Eggs were microinjected with a solution of linear DNA containing MThGH genes.

[b]The number of animals (fetuses, stillborns and neonates) resulting from injected ova. Numbers in parentheses are the number of animals produced/total eggs injected.

[c]The number of animals that incorporated the injected DNA. Numbers in parentheses are the number of MThGH animals/total number of animals produced.

[d]The number of animals containing MThGH mRNA. Numbers in parentheses are the number of MThGH animals expressing the gene/total number of MThGH animals analyzed. Sixteen rabbits were analyzed for mRNA. ND = not determined.

[e]Data for mice are from Brinster et al. (1985).

[f]Integration efficiency for pigs based on combined data from female recipients bearing injected eggs only or injected and control eggs (Hammer et al., 1985c).

[g]Pigs containing detectable plasma human growth hormone. Plasma from 18 animals was analyzed for hGH.

TABLE 4. CHARACTERISTICS OF MThGH TRANSGENIC RABBITS AND SHEEP

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Sheep	693-1[d]	1	ND[e]	ND

[a]Animals without identified gender were sacrificed as fetuses.

[b]Liver MThGH mRNA was quantitated.

[c]Male.

[d]Female.

[e]ND = not determined.

able levels of hGH at birth and by 90 d of age hGH had increased substantially in five of these pigs (table 5). In addition, five of the six pigs that were negative for hGH at birth had low but detectable levels at 90 d of age. In MThGH mice, hGH mRNA is present in fetal liver as early as d 13 of gestation (R. Palmiter, unpublished observation). However, the developmental pattern of MThGH expression in mice and pigs has not been fully explored.

Effect of Foreign GH on Growth. Many of the founder transgenic mice bearing MT-GH fusion constructs show up to a fourfold enhancement in growth rate during the maximum growth phase. Enhanced growth is evident by 3 wk of age and growth rates by 11 wk have begun to plateau at about twice normal body size (Palmiter et al., 1983; Hammer et al., 1984a). Although the MT promoter is responsive to heavy metals and GH mRNA levels increase as a result of metal induction, the additional GH does not further enhance growth (Hammer et al., 1984b). Thus, in mice the constitutive activity of the MT promoter provides enough foreign GH to stimulate growth maximally.

The effects of hGH on growth of transgenic rabbits has not been evaluated. The animal that had detectable hGH levels had malocculusion, which impaired food consumption. In pigs, hGH in the serum of expressing animals did not increase weight gain (table 6). Transgenic offspring and full-sib littermate controls will need to be generated to determine precisely the effect of hGH on growth rates and feed efficiency. However, these preliminary data indicate that hGH will not dramatically alter growth performance. Such findings may not be surprising since injections of recombinantly derived hGH had only a weak growth-promoting effect in pigs (Baile et al., 1983). In contrast, injections of porcine (p) GH (22 $\mu g \cdot kg^{-1}$ body $wt \cdot d^{-1}$) into pigs stimulated growth by 10% and improved feed efficiency by 4% (Chung et al., 1985). Larger doses of pGH (70 $\mu g \cdot kg^{-1}$ body $wt \cdot d^{-1}$) further enhance swine growth rates and feed efficiency (21%) and the

TABLE 5. CHARACTERISTICS OF MThGH TRANSGENIC PIGS

Animal	Sex[a]	Gene copy, no./cell	hGH mRNA, molecules/cell	Immunoassayable hGH (ng/ml)	
				Birth	90 d
11-2	M	1	0	40	20
16-9	M	1	5	65	72
21-4	M	1	2	NEG[b]	2
21-5	F	1	0	NEG	NEG
20-2	M	2	4	40	337[c]
25-4	F	2	0	NEG	2
16-3	F	3	0	NEG	32
17-4[f]	F	3	0	NEG	
18-3[d]	F	3	6	60	
23-8	F	7	41	730	2,435
16-8[e]	F	10	18	53	
25-2	F	17	0	108	584
10-4	F	23	0	NEG	3
22-1[e]	F	50	24	56	
7-3[g]	F	90	12	80	
20-8	M	110	1	2	113
3-2	M	330	26	NEG	3
3-6	M	490	53	17	48

[a] M = male, F = female.

[b] NEG = negative.

[c] Plasma sample was taken at 60 d of age.

[d] Sacrificed at birth.

[e] Stillborn.

[f] Died at 6 wk of age.

[g] Died at 3 wk of age.

TABLE 6. GROWTH PERFORMANCE OF MThGH TRANSGENIC PIGS[a]

Item	Control pigs[b]	MThGH pigs[c]	
		Non-expressors	Expressors
No. of animals	7	5	5
Avg daily gain, kg	.78 ± .03	.69 ± .02	.63 ± .04

[a]The experimental period began from an initial body weight of 30 kg and terminated at 90 kg. Values are means ± SE. Pigs were fed a corn-soybean diet containing approximately 16% protein.

[b]Control pigs were non-transgenic littermates of experimental animals.

[c]MThGH transgenic pigs are founder animals listed in table 5. Expression is based on the presence of immunoassayable plasma hGH.

linearity of such responses suggests the possibility that even higher doses may further stimulate growth performance (Rebhun et al., 1985). Thus, introducing the porcine GH gene into pigs under the control of the mouse or human MT promoter or an alternative promoter may provide sufficient plasma pGH levels to enhance growth.

Alternatively, genes coding for other peptides involved in growth regulation may be introduced into domestic species. Expression of a mouse MT-human growth hormone-releasing factor (hGRF) gene in transgenic mice increases somatic growth by elevating endogenous GH levels (Hammer et al., 1985b); in steers injections of hGRF increase serum GH concentrations (Mosley et al., 1985). Genes coding for somatomedins or insulin-like growth factors (IGF) I and II have been cloned (Jansen et al., 1983; Bell et al., 1984; Ullrich et al., 1984) and, while the consequences of IGF expression from foreign genes in mice have not yet been explored, it seems possible that enhanced growth performance will result. Such possibilities and applications in livestock await further investigation.

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APPENDIX I

Integration, expression and germ-line transmission of growth-related genes in pigs

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Summary. We have produced transgenic pigs that harbour structural genes for bovine and human growth hormone (bGH and hGH) ligated to a mouse metallothionein-I (MT) promoter, human growth hormone-releasing factor (hGRF) ligated to the MT or mouse albumin (ALB) promoter, and human insulin-like growth factor-I (hIGF-I) ligated to MT promoter. From 0·31 to 1·03% of microinjected ova developed into transgenic pigs with the various fusion genes. Foreign GH was present in plasma of 61% of the MT–hGH and 89% of the MT–bGH transgenic pigs. Two of 7 pigs with MT–hGRF and all 3 ALB–hGRF transgenic pigs had high concentrations of GRF in their plasma, but plasma concentrations of porcine GH (pGH) were not higher in GRF transgenic pigs than in littermate control pigs. In contrast, plasma concentrations at birth ranged from 3 to 949 ng hGH/ml for MT–hGH transgenic pigs and 5 to 944 ng bGH/ml for MT–bGH transgenic pigs. Presence of the foreign GH depressed endogenous pGH to non-detectable levels. In MT–bGH transgenic pigs, plasma IGF-I was elevated more than 2-fold, plasma glucose was elevated about 30 mg/dl, and plasma insulin was 20-fold higher than in littermate or sibling control pigs. Two lines of pigs expressing the MT–bGH transgene gained 11·1% and 13·7% faster, and were 18% more efficient in converting feed to body weight gain than were sibling control pigs. Expression of the MT–bGH transgene caused a marked repartitioning of nutrients from subcutaneous fat into other carcass components, including muscle, skin, bone and certain organs. The persistent excess hGH or bGH in transgenic pigs was detrimental to general health; lameness, lethargy and gastric ulcers were the most prevalent problems. Gilts that expressed the hGH or bGH transgenes were anoestrous. Germ-line transmission was obtained in 4 of 5 expressing transgenic boars and 4 of 5 non-expressing transgenic boars and gilts. From 2% to 73% of progeny inherited a transgene from founder transgenics. All transgenic progeny of MT–hGH, MT–bGH and MT–hGRF founder males expressed the transgene if their sire also expressed the gene. The concentration of bGH or hGH in plasma of transgenic progeny was similar to the concentration present in the founder transgenic.

Keywords: pig; transgenic; growth; gene transfer; gene expression

Introduction

The dramatic achievements in molecular biology during the past decade and the development of micromanipulation for early-stage embryos provided the combined capabilities for introducing

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cloned genes into the mouse genome in 1980 (see review by Brinster & Palmiter, 1986; Palmiter & Brinster, 1986). The transfer of genes was immediately recognized as an important scientific achievement, but the subsequent creation of the 'super' mouse by the transfer of a rat growth hormone (rGH) gene provided the convincing evidence that demonstrated the potential offered by gene transfer (Palmiter *et al.*, 1982).

The general strategy used by Palmiter *et al.* (1982) with the mouse, which we subsequently applied to pigs (Hammer *et al.*, 1985c; Pursel *et al.*, 1987, 1989b), was to insert foreign growth-regulating genes under control of a heterologous promoter to direct the production of peptides in ectopic tissues and evade the usual regulatory system for these peptides.

The purpose of this report is to review our recent progress on the gene integration rate, the frequency, level, and consequences of transgene expression, and the subsequent germ-line transmission of several growth-regulating genes in pigs.

Transfer of fusion genes

Fusion genes

Growth-related fusion genes that we introduced into the genome of pigs are shown in Fig. 1. A mouse metallothionein (MT) promoter was used to direct each of the four structural genes. In addition, a mouse albumin (ALB) promoter was used with human growth hormone-releasing factor (hGRF). The human and bovine growth hormone (hGH and bGH) structural genes were cloned from genomic DNA libraries and contained intron sequences (Hammer *et al.*, 1985a). The hGRF structural gene was a fusion of genomic and cDNA sequences and contained only one intron (Hammer *et al.*, 1985b). The human insulin-like growth factor-I (hIGF-I) structural gene was composed of a cDNA sequence and a signal peptide from a rat somatostatin gene.

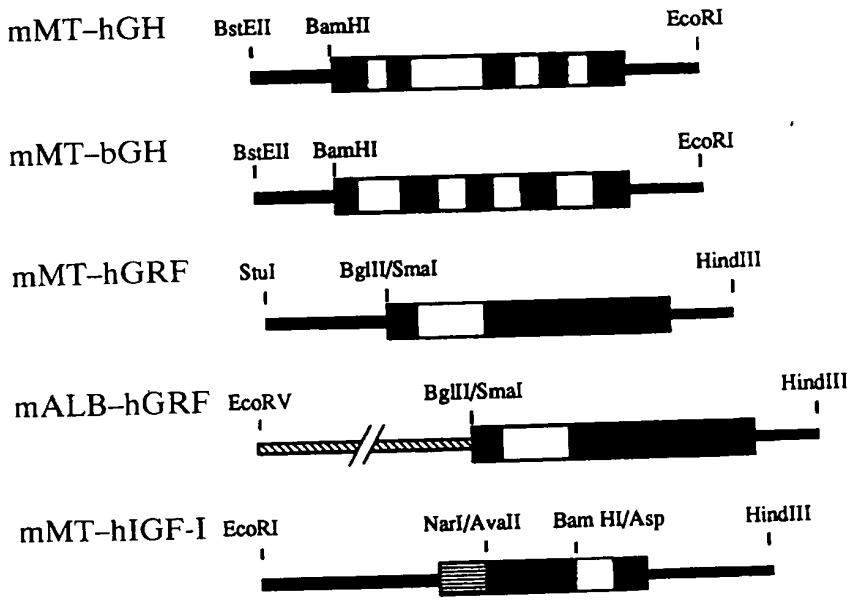

Fig. 1. Diagram of fusion genes that were excised from plasmids and microinjected into pig ova. Selected restriction sites for each construct are shown. The promoter and flanking sequences are a stippled box, exons are solid, and introns are open boxes. The horizontal-lined box in mMT–hIGF-I represents a signal peptide from a rat somatostatin gene.

Microinjection

The only technique that has so far produced transgenic pigs is the microinjection of genes into a pronucleus of 1-cell ova or nuclei of 2-cell ova. Our initial attempts to microinject genes into the pronuclei of pig ova were impeded because the opacity of the cytoplasm of pig ova precluded visualization of pronuclei. However, the lipid granules in the cytoplasm can be displaced to one end of pig ova by centrifugation at 15 000 *g* for 5 min, leaving pronuclei in the equatorial segment of the cytoplasm (Wall *et al.*, 1985). The pronuclei are then visible for microinjection with the aid of interference-contrast microscopy.

Synchrony of donors and recipients

Oestrous cycles of all donor and many recipient gilts or sows were hormonally synchronized by treatment with altrenogest or methallibure. Donor gilts also were injected with pregnant mares' serum gonadotrophin (PMSG) to induce superovulation and subsequently were injected with human chorionic gonadotrophin (hCG) 78–82 h after the PMSG injection to induce ovulation. Hormonally synchronized recipients received only the hCG injection.

The synchrony of the oestrous cycles of the donor and recipient females influenced the farrowing rate obtained after the transfer of microinjected ova. During one experiment, ova were either transferred into recipients that received hCG synchronous with donor gilts or transferred into recipients that received hCG 11–17 h after the donors (delayed). The farrowing rate of recipient gilts with delayed ovulation was significantly higher than the farrowing rate of recipients ovulating in synchrony with the donor gilts (Table 1).

Table 1. Pregnancy rate and litter size of recipient gilts ovulating in synchrony with or 11–17 h after donors of microinjected ova

Ovulation in recipient	No. of recipients	Pregnant		Pigs or fetuses	
		No.	%	Mean	s.e.m.
Synchronous	21	9	43*	8·6	0·83
Delayed	25	18	72*	8·2	0·59

*$P < 0{\cdot}05$ ($\chi^2 = 3{\cdot}99$).

Whenever possible thereafter, we attempted to use recipient gilts that ovulated 11–17 h after the donors. However, during the course of our studies, if insufficient numbers of gilts with delayed ovulation were available, or if recipients that were hormonally treated did not exhibit oestrus or did not ovulate, then naturally cyclic gilts or sows were used as recipients if they had begun oestrus on the morning after the donors received an injection of hCG and were still in oestrus on the following morning. The results that are summarized in Table 2 show that the farrowing rate of the naturally cyclic gilts and sows was similar to that of synchronous recipients (Table 1) and was significantly lower than for the recipients with delayed ovulation. Possibly, these results are influenced by the 8–14-h period of in-vitro incubation to which our microinjected ova were subjected to accommodate their transport from Beltsville after recovery to Philadelphia for microinjection and subsequent return to Beltsville for transfer into recipients.

Gene integration

Integration efficiency

The overall efficiency of integrating growth-related transgenes into the pig genome is quite low. Only 8·3% of all transferred microinjected ova were represented by offspring at birth (Table 3).

Table 2. Pregnancy rate and litter size of recipient gilts ovulating naturally or 11–17 h after donors of microinjected ova

Ovulation in recipient	No. of recipients	Pregnant		Pigs or fetuses	
		No.	%	Mean	s.e.m.
Delayed	138	93	67*	6·9	0·30
Natural†	100	43	43*	6·5	0·44

*$P < 0{\cdot}001$ ($\chi^2 = 14{\cdot}09$).
†In oestrus on morning after donors received injection of hCG.

Integration efficiency varied from 0·31% to 1·03% when expressed as the percentage of injected ova that resulted in transgenic pigs (Table 3). Our efficiencies of gene integration are similar to those reported by Brem *et al.* (1985, 1988) and Ebert *et al.* (1988) but are slightly lower than efficiencies of 1·42% reported by Vize *et al.* (1988) and 1·73% reported by Polge *et al.* (1989). In contrast, transfer of the same fusion genes into mice resulted in an efficiency averaging in excess of 3% (Brinster *et al.*, 1985).

Table 3. Efficiency of transferring growth-regulating genes into pigs (after Pursel *et al.*, 1989a, b)

Fusion gene	No. of ova injected	No. of recipient gilts	Gilts pregnant		Offspring		Transgenic		Expressing transgene	
			No.	%	No.	%	No.	%	No.	%
MT–hGH	2035	64	37	58	192	9·4*	20	0·98†	11/18	61‡
MT–bGH	2330	49	24	49	150	6·4	9	0·41	8	89
MT–hGRF	2236	66	35	53	177	7·9	7	0·31	2	29
ALB–hGRF	968	32	20	62	108	11·2	5	0·52	3/3	100
MT–hIGF–I	387	13	5	38	34	8·8	4	1·03	1/2	50

*Percentage of injected ova resulting in offspring.
†Percentage of injected ova resulting in a pig with gene integration.
‡Percentage of transgenic pigs expressing the fusion gene.

The number of transgene copies integrated per cell in MT–hGH transgenic pigs varied from 1 to 490 (Hammer *et al.*, 1985c), <1 to 28 for MT–bGH (Miller *et al.*, 1989), <1 to 15 for MT–pGH (Vize *et al.*, 1988), and <1 to >10 for prolactin–bGH (Polge *et al.*, 1989) with most integrations probably occurring at a single locus. Southern blot analysis showed that transgenic pigs with MT–hGH contained intact copies of the fusion gene with many orientated in tandem head-to-tail arrays and some in a head-to-head configuration (Hammer *et al.*, 1985c). Only one of four MT–pGH transgenic pigs studied contained the transgene organized in the head-to-tail array (Vize *et al.*, 1988).

Expression of integrated transgenes

Incidence of expression

Integrated genes were expressed in 11 of 18 (61%) MT–hGH transgenic pigs and 8 of 9 (89%) MT–bGH transgenic pigs (Table 3). Only 2 of 7 (29%) MT–hGRF pigs expressed the transgene, which was similar to the incidence of expression of 1 of 7 (14%) transgenic lambs with the same

gene (Rexroad *et al.*, 1989). In contrast, 11 of 14 (79%) transgenic mice expressed MT–hGRF (Hammer *et al.*, 1985b). Reasons for this discrepancy in incidence of expression among species are unknown.

Failure to express the transgene may be the result of integration in an inactive chromosomal locus or alteration of gene sequences during the integration process. In some cases, the presence of introns in the structural region may greatly influence whether the gene is expressed. Brinster *et al.* (1988) found in mice that genes without introns were expressed with a lower frequency and at a lower level than the same genes with introns.

Site of transgene expression

Amounts of bGH mRNA in several organs of two lines of MT–bGH transgenic pigs were determined by solution hybridization (Table 4). The MT promoter directed expression of fusion genes to the expected tissues in transgenic pigs. However, the concentrations of GH mRNA in pig tissues were considerably lower than in tissues of transgenic mice harbouring the same fusion gene (Hammer *et al.*, 1985a). While the quantity of bGH mRNA varied in both lines, pigs in the 31-04 line had high levels of bGH mRNA in liver, kidney, adrenal and pancreas, which was consistent with the high concentration of bGH found in their plasma (Table 4). In contrast, founder transgenic pig 37-06 and his transgenic descendants had very low but detectable levels of bGH mRNA in 1–4 of the organs and low concentrations of bGH were present in their plasma.

Table 4. Plasma bGH and bGH mRNA values for several organs of two lines of pigs expressing a MT–bGH transgene

Pig line	Generation	Plasma bGH (ng/ml)	bGH mRNA* (molecules/cell)						
			Liver	Kidney	Heart	Gonad	Lung	Adrenal	Pancreas
Line 37-06									
37-06	G0	45	0	150	360	50	0	0	0
67-06	G1	111	45	0	0	0	0	14	45
67-07	G1	70	31	0	0	55	0	13	285
136-01	G2	183	56	0	0	NA†	70	0	65
136-02	G2	38	43	23	0	NA	0	14	75
136-03	G2	29	0	0	0	NA	12	0	55
137-02	G2	39	0	0	0	21	20	5	12
140-02	G2	53	0	0	0	0	14	0	0
Line 31-04									
115-14	G1	1089	1720	2320	18	500	0	975	70
182-07	G2	822	690	110	NA	203	140	965	510
185—03	G2	2138	870	1170	15	300	57	2250	2150

*Total nucleic acids were extracted from homogenized tissues and mRNA was measured by solution hybridization using a ^{32}P-labelled oligonucleotide. Molecules per cell were calculated by assuming 6·4 pg DNA per cell. 0 = less than twice background.

†NA = not assayed.

Level of gene expression

The level of gene expression varied greatly among transgenic pigs that had integrated the same fusion gene. Plasma concentrations at birth ranged from 3 to 949 ng hGH/ml and 5 to 944 ng bGH/ml in MT–hGH and MT–bGH transgenic pigs, respectively (Hammer *et al.*, 1985c; Miller *et al.*, 1989). Plasma concentrations of hGH were unrelated to the number of gene copies per cell in MT–hGH transgenic pigs (Hammer *et al.*, 1985c). However, Miller *et al.* (1989) found that, in transgenic pigs with MT–bGH, the concentration of bGH in plasma both at birth or at 30–180 days of age was

positively correlated ($P < 0{\cdot}05$) with the number of gene copies per cell. This relationship is evident in Fig. 2.

The concentration of GRF in plasma of transgenic pigs with MT–hGRF or Alb–hGRF was 130–380 pg/ml and 400–8000 pg/ml, respectively (Pursel *et al.*, 1989a, b). These values are 10- to 500-fold higher than concentrations of GRF in plasma of littermate control pigs. However, most of the assayable GRF in plasma of the MT–hGRF pigs was 3–44 metabolite rather than the native peptide (1–44), which may explain why the concentration of pGH in plasma was not elevated in the transgenic compared to the littermate controls (Pursel *et al.*, 1989a).

In transgenic mice harbouring fusion genes with the MT promoter, concentrations of hGH and bGH in plasma were frequently elevated more than 10-fold after zinc was added to their drinking water (Palmiter *et al.*, 1983; Hammer *et al.*, 1985a). In contrast, addition of 1000–3000 p.p.m. zinc to the feed of transgenic pigs resulted in little more than a doubling of the bGH concentration in plasma (Pursel *et al.*, 1990). Several pigs with a prolactin (PRL)–bGH transgene released bGH in an episodic fashion after pigs were injected with thyrotrophin-releasing hormone (TSH) or after infusion of a dopamine antagonist, sulpiride (Polge *et al.*, 1989). Both studies with pigs demonstrated that the promoter sequences of fusion genes can respond to induction.

Physiological consequences of transgene expression

The rapid rate of growth and large body size of mice that expressed GH transgenes (Palmiter *et al.*, 1982, 1983) created the expectation that the rate of growth might be greatly enhanced if the same GH transgenes were transferred into swine. The founder population of MT–hGH and MT–bGH transgenic pigs did not meet this expectation even though hGH and bGH were biologically active (Hammer *et al.*, 1986; Pursel *et al.*, 1987, 1989b). Pigs expressing the hGH transgene rarely had detectable concentrations of plasma pGH (Miller *et al.*, 1989), which indicates that the negative feedback mechanism was functioning. Furthermore, insulin-like growth factor-I (IGF-I) concentrations in hGH and bGH expressing transgenic pigs were considerably higher than in littermate control pigs (Fig. 3).

The effects of bGH gene expression on concentrations of glucose and several metabolic hormones in the plasma are summarized in Table 5. In comparison to littermate controls, pigs expressing MT–bGH had significantly elevated concentrations of glucose and insulin, significantly lower values of thyroxin and prolactin, and concentrations of cortisol and triiodothyronine were similar. These results are comparable to those reported for pigs injected daily with exogenous pGH, which had average increases in serum glucose ranging from 8% to 48% and concentrations of serum insulin that were 2- to 7-fold higher than in control pigs (Campbell *et al.*, 1988, 1989; Evock *et al.*, 1988). Additionally, Ebert *et al.* (1988) reported that a transgenic pig expressing rat GH had glucosuria and consistently had serum glucose concentrations more than 3-fold higher than control pigs.

The founder MT–hGH and MT–bGH transgenic pigs were fed a diet containing only 16% protein that may not have provided sufficient protein for them to gain weight faster than their littermate controls. Recent studies using pigs injected with exogenous pGH indicate that maximal growth rate is attained only if the diet contains adequate protein and, particularly, lysine (Goodband *et al.*, 1988; Newcomb *et al.*, 1988). Subsequently, we increased the levels of dietary protein and lysine during the 30–90-kg growth period and found that the G2 and G3 progeny of MT–bGH transgenic founder 37-06 gained weight 11·1% faster, and G2 progeny of 31-04 founder gained weight 13·7% faster than did sibling control pigs (Table 6).

In comparison to littermate or sibling control pigs, food intake was depressed 20% in MT–bGH founders (Pursel *et al.*, 1989b) and 17% in G2 progeny of MT–bGH 37-06 line of transgenic pigs fed *ad libitum* (Table 6). These results are comparable to a 14% and 17% depression in food intake reported for pigs injected with pGH (Campbell *et al.*, 1988; Evock *et al.*, 1988). The appetite depression that accompanies elevated GH in pigs is probably a major factor that inhibits pigs from

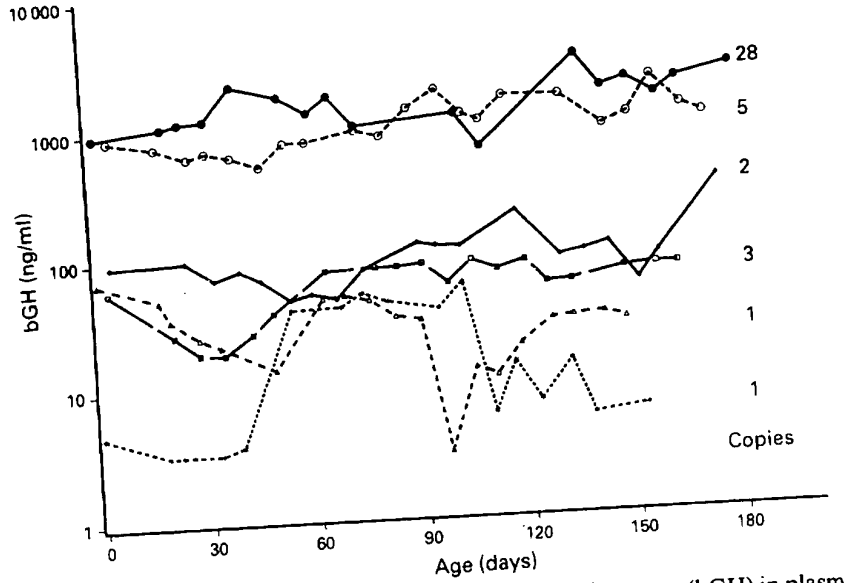

Fig. 2. Each line identifies the concentration of bovine growth hormone (bGH) in plasma for a single MT–bGH founder transgenic pig. The number of integrated gene copies per cell for each pig is indicated at the right (after Miller *et al.*, 1989).

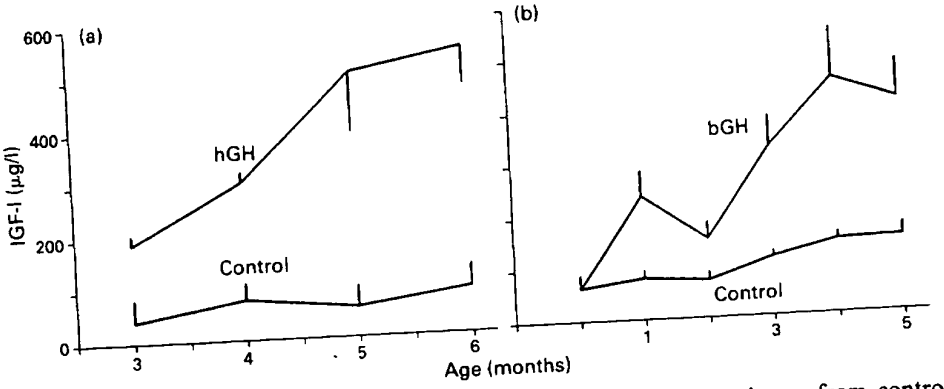

Fig. 3. Concentrations of insulin-like growth factor-I (IGF-I) in (a) plasma from control (N = 3) and MT–hGH expressing (hGH; N = 3) pigs from 3 to 6 months of age and (b) plasma from control (N = 6) and MT–bGH expressing (bGH; N = 6) pigs from 0 to 5 months of age. Values are means + s.e.m. (After Miller *et al.*, 1989.)

achieving greatly accelerated growth rates. In contrast, growth is dramatically increased in mice expressing GH transgenes or in rats with a GH-secreting tumour, which have greatly enhanced food intake (McCusker & Campion, 1986).

MT–bGH founder transgenic pigs were 16% more efficient (Pursel *et al.*, 1989b), and G2 progeny of MT–bGH 37-06 line transgenic pigs were 18% more efficient in converting food into body weight gain than were littermate or sibling controls (Table 6). These results are quite similar to improved food efficiencies of 23% (Campbell *et al.*, 1988) and 25% (Evock *et al.*, 1988) reported for pigs injected with exogenous pGH in comparison to littermate controls.

Table 5. Glucose and metabolic hormone concentrations in plasma of MT–bGH transgenic and littermate control pigs

Item	Control pigs	Transgenic pigs	Significance*
Glucose† (mg/dl)	72 ± 5 (10)	109 ± 13 (10)	$P = 0.011$
Cortisol‡ (ng/ml)	39 ± 9 (6)	37 ± 7 (8)	$P = 0.84$
Insulin† (pg/ml)	24 ± 4 (10)	480 ± 118 (10)	$P = 0.001$
Prolactin† (ng/ml)	3·9 ± 0·5 (10)	2·3 ± 0·4 (10)	$P = 0.021$
T_3‡ (ng/ml)	1·2 ± 0·3 (6)	1·3 ± 0·2 (7)	$P = 0.68$
T_4‡ (ng/ml)	49 ± 4 (6)	29 ± 3 (7)	$P = 0.003$

Values are mean ± s.e.m. for the no. of pigs indicated in parentheses. T_3 = triiodothyronine; T_4 = thyroxine.
*Comparison by least squares analysis of variance.
†Blood collected after overnight fast.
‡Blood collected from cannulated pigs.

Table 6. Average daily gain and feed efficiency of MT–bGH transgenic pigs (30–90 kg body weight) (after Pursel *et al.*, 1989b)

Line	Group*	Average daily gain (g)	Kg feed/kg gain
37-06†	Control	813 ± 17 (23)	2·99 ± 0·12 (8)
	Transgenic	903 ± 23 (13)	2·46 ± 0·16 (5)
		$P = 0.002$	$P = 0.026$
31-04‡	Control	869 ± 43 (7)	ND§
	Transgenic	988 ± 62 (7)	ND§
		$P = 0.15$	

Values are mean ± s.e.m. for the no. of animals in parentheses, and were compared by least-squares analysis of variance.
*Pigs were fed corn-soybean diet containing 18% crude protein plus 0·25% lysine.
†G2 and G3 progeny of founder 37-06.
‡G2 progeny of founder 31-04.
§Not determined because pigs were group fed.

Elevated concentrations of GH in pigs expressing MT–hGH and MT–bGH transgenes have produced marked repartitioning of nutrients away from subcutaneous fat and into other carcass components, including muscle, skin, bone and certain organs. Slaughter measurements or ultrasonic estimates of backfat thickness at the 10th rib of MT–hGH and MT–bGH transgenic pigs at ~90 kg body weight averaged 7·0 mm and 7·9 mm, respectively, while littermate control pigs averaged 18·5 and 20·5 mm, respectively ($P < 0.01$ in each case; Hammer *et al.*, 1986; Pursel *et al.*, 1989b). Additionally, backfat measurements do not adequately reflect the lack of subcutaneous fat in transgenic pigs because the skin over the 10th rib was about 1 mm thicker for transgenic pigs than for control pigs (V. G. Pursel & M. B. Solomon, unpublished data).

Pigs expressing either the MT–hGH or MT–bGH transgene exhibited several notable health problems, including lameness, susceptibility to stress, gastric ulcers, parakeratosis, lethargy, anoestrus in gilts, and lack of libido in boars (Pursel *et al.*, 1987, 1989b). Pathology in joints, characteristic of osteochondritis dissecans, was also reported in a pig expressing an rGH transgene (Ebert *et al.*, 1988) and in some pigs treated with exogenous pGH for 57 days (Evock *et al.*, 1988). In contrast, no increase in the incidence of these pathological conditions was observed in

non-expressing MT–hGH or MT–bGH transgenic pigs (Pursel *et al.*, 1987) or in transgenic pigs that expressed only low levels of bGH (Polge *et al.*, 1989).

Many of the health problems observed in pigs exposed to high concentrations of GH are quite prevalent in the general pig population but at a lower incidence and with less severity. Several necropsy surveys indicate that gastric ulcers were present in 10–30% of market hogs at slaughter (O'Brien, 1986), and up to 90% of rapidly growing pigs have lesions of osteochondrosis, which leads to degenerative joint disease in certain pigs and is the major cause of lameness in swine (Reiland *et al.*, 1978; Carlson *et al.*, 1988). Additional investigation is required to determine whether these ailments would be less prevalent in pigs expressing GH transgenes if the genetic base was rigidly selected for a low incidence of these conditions.

Reproductive capacity was seriously impaired in pigs expressing either the MT–hGH or MT–bGH transgene. Gilts failed to exhibit oestrus, and their ovaries were devoid of corpora lutea or corpora albicantia when examined at necropsy (V. G. Pursel, unpublished data). Boars totally lacked libido; therefore, spermatozoa were recovered by electroejaculation or were flushed from the epididymis at necropsy to use for artificial insemination to obtain germ-line transmission of the transgene (Pursel *et al.*, 1987).

A major difference between a transgenic pig with elevated GH and a normal pig injected daily with exogenous GH is that, in the latter case, GH is elevated episodically, while in the transgenic pig, GH is elevated continuously (Miller *et al.*, 1989). We suggest that continuous exposure to elevated concentrations of GH contributes to the multiple health problems observed in our MT–hGH and MT–bGH transgenic pigs and also may prevent them from growing to their full potential. There is evidence for the latter in the rat since Robinson & Clark (1989) reported that rats infused continuously with a high concentration of GH do not attain the maximal rate of growth achieved in rats injected once daily. Use of promoters that permit expression of GH fusion genes only during the rapid growth phase or promoters that can induce the release of large episodic doses of GH may be essential to achieve only the positive aspects of elevated GH for pigs. Future research on growth regulation with transgenic pigs will most certainly be directed toward this goal, along with investigation of the potentials of IGF-I, GH receptors, and other structural genes involved in growth.

Transmission of transgenes

If economically important transgenic livestock become available, it will be essential that these animals have the ability to transmit the transgene to progeny in a predictable manner. We have tested transgenic founder pigs harbouring three different transgenes for transmission to progeny. So far, 8 of 10 transgenic pigs successfully transmitted the transgene to one or more progeny (Table 7). Many of the pigs transmitted their transgene to about 50% of the progeny as expected with simple Mendelian inheritance. The MT–hGH sow and MT–bGH transgenic boar that failed to transmit the transgene to their progeny were probably mosaic for the gene, with integration only in somatic cells (Table 7). In another MT–hGH boar, integration was evidently mosaic in the germ line since the transgene was only transmitted to 1 of 52 progeny (Pursel *et al.*, 1987). Mosaicism in the germ line was found in 25–36% of transgenic mice produced by microinjection (Wilkie *et al.*, 1986).

We obtained germ-line transmission from expressing and non-expressing transgenic pigs. All transgenic progeny sired by boars that expressed MT–hGH, MT–bGH or MT–hGRF transgenes have also expressed the transgene (Pursel *et al.*, 1987; Table 7). One of 4 MT–hGH non-expressing transgenic founders sired 17 transgenic progeny of which two expressed the transgene as was evident from concentrations of less than 10 ng hGH/ml plasma (V. G. Pursel, K. F. Miller & D. J. Bolt, unpublished data). The transgenic progeny of the other 3 non-expressing founder pigs did not express the transgene.

Table 7. Transmission of growth-regulating transgenes from transgenic founder pigs to progeny

Fusion gene	Founder	Sex	No. of gene copies	Expressing	No. of litters	Progeny				
						No. born	Transgenic		Expressing	
							No.	%	No.	%
MT–hGH	3-02	M	330	No	6	52	17	33	2	12
	10-04	F	23	No	1	12	5	42	0	0
	11-02	M	1	Yes	1	13	3	23	3	100
	16-03	F	3	No	3	33	0	0	0	0
	21-04	M	1	No	6	52	1	2	0	0
	25-04	F	2	No	1	8	5	63	0	0
MT–bGH	29-01	M	5	Yes	4	36	0	0	0	0
	31-04	M	28	Yes	3	19	6	32	6	100
	37-06	M	3	Yes	1	11	8	73	8	100
MT–hGRF	86-04	M	100	Yes	3	20	11	55	10/10	100

While concentrations of hGH or bGH in plasma varied widely from one founder to the next (Miller *et al.*, 1989; Fig. 2), successive generations of two lines of MT–bGH transgenic pigs maintained quite consistent levels of expression (Pursel *et al.*, 1989b). Transgenic pigs in the MT–bGH 31-04 line maintained plasma concentrations of about 1300 ng bGH/ml plasma over 3 generations while pigs in the MT–bGH 37-06 line maintained concentrations of about 85 ng bGH/ml plasma over 4 generations (Pursel *et al.*, 1989b). These findings provide evidence that fusion genes can become stably integrated into the pig genome and usually function in descendants in the same manner as they did in the founder transgenic.

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APPENDIX J

TRANSFERRIN- AND ALBUMIN-DIRECTED EXPRESSION OF GROWTH-RELATED PEPTIDES IN TRANSGENIC SHEEP[1]

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ABSTRACT

Chimeric genes containing either the mouse transferrin (Trf) enhancer/promoter fused to the structural sequences encoding bovine growth hormone (GH) or the mouse albumin (Alb) enhancer/promoter fused to the gene for human growth hormone-releasing factor (GRF) were microinjected into sheep zygotes. A low percentage of resulting transgenic sheep chronically expressed the respective genes, resulting in elevated plasma concentrations of circulating GH or GRF, respectively. Growth hormone-releasing factor expression induced elevated plasma levels of endogenous GH production. In addition, elevated levels of circulating insulin-like growth factor-I were observed in the bovine GH-expressing Trf transgenic sheep. Growth of these founder transgenic sheep relative to controls were not enhanced. In part, this may be due to the development of the diabetic condition exhibited by both transgenic groups. These results demonstrate that the mouse Trf and Alb enhancer/promoters are active in sheep and suggest that alternate strategies for expressing growth-related genes may be required to modulate growth in sheep.

Key Words: Transgenics, Sheep, Genes, Growth, Hormones, Hypothalamic Releasing Hormones

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Introduction

Genetic improvement of livestock by selection requires many years. Transfer of foreign genes into the germ line may permit rapid improvement of production traits of livestock (Hammer et al., 1985c; Pursel et al., 1989). Genetic engineering strategies for improving growth employ transgenes with regulatory regions from genes not expressed in the pituitary to allow expression of growth-related gene products at ectopic sites. Ectopic expression of foreign growth hormone (GH) genes or human growth hormone-releasing factor gene (GRF) regulated by the mouse metallothionein-I (MT) promoter in transgenic mice resulted in chronically elevated levels of circulating foreign GH and endogenous GH, respectively (Palmiter et al., 1982, 1983; Hammer et al., 1985a,b). Growth rates and mature body size increased compared with nontransgenic littermate mice. Ectopic expression of foreign MT-GH genes in transgenic sheep and pigs increased plasma levels of foreign GH (Hammer et al., 1985a; Pursel et al., 1987; Ebert et al., 1988; Vize et al., 1988; Rexroad et al., 1989; Ward et al., 1989). Ectopic expression of MT-GRF fusion genes increased circulating concentrations of endoge-

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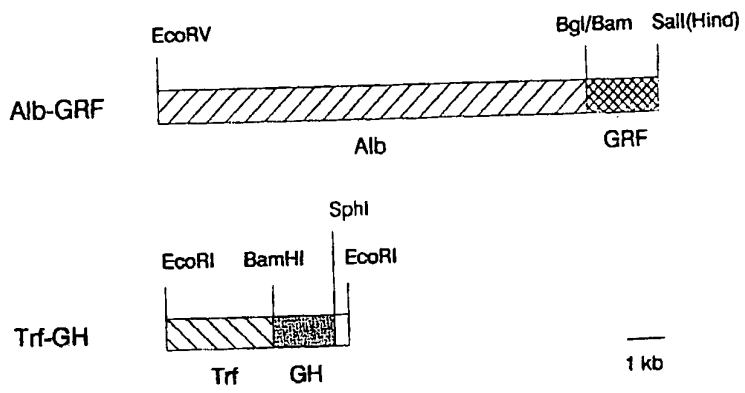

Figure 1. Fusion gene used for production of transgenic sheep. Slanted lines represent the regulatory regions of fusion genes. Cross-hatched and stippled regions represent the structural gene elements. See "Gene Construction" in materials and methods for details.

nous GH in transgenic sheep but not in pigs (Pursel et al., 1989; Rexroad et al., 1989). Growth efficiency improved, whereas backfat and total body lipid decreased in transgenic pigs chronically expressing bovine GH (Pursel et al., 1989). Carcass fat content decreased in transgenic lambs expressing ovine GH genes (Ward et al., 1989). Pathological conditions including lameness and joint pathology, gastric ulcers, anestrus, and early mortality (Pursel et al., 1987, 1989; Ebert et al., 1989) in transgenic pigs and early mortality in transgenic sheep (Rexroad et al., 1989; Ward et al., 1989) resulted from overproduction of GH.

Because of the pathologies associated with overproduction of GH in transgenic sheep with MT-GH or MT-GRF transgenes, we selected the mouse transferrin (Trf) promoter for a GH transgene (Trf-GH) and albumin (Alb) enhancer/promoters for a GRF transgene (Alb-GRF) for experiments in transgenic sheep. Both transgenes increased circulating levels of GH in sheep. Transferrin-growth hormone increased insulin-like growth factor I (IGF-I) in transgenic lambs. Both types of expressing transgenic sheep did not thrive and developed a diabetes-like condition.

Experimental Procedure

Gene Construction. To generate the Trf-GH fusion gene (Figure 1) a mouse transferrin-human growth hormone fusion gene was constructed by fusing the 3-kb *Eco*RII-*Bam*HI fragment containing the mouse Trf promoter and enhancer to the *Bam*HI site of human GH (Idzerda et al., 1989). The *Bam*HI-*Sph* I fragment of bovine GH was substituted for the comparable fragment of human GH, leaving about .3 kb of human GH 3′ flanking sequence. A 5.1-kb *Eco*RI fragment was isolated for microinjection. The Alb-GRF fusion gene (Figure 1) was generated by ligating a 12-kb *Eco*RV-*Bam*HI fragment containing the mouse Alb promoter and enhancer (Pinkert et al., 1987) to the 1.7-kb human GRF minigene *BGl*II-*Hind*III fragment (Hammer et al., 1985a) in which the *Hind*III site had been converted to *Sal* I. The 13.7-kb *Eco*RV-*Sal* I fragment was isolated for microinjection.

Generation of Transgenic Sheep. Zygotes were collected from superovulated parous ewes of mixed breeding as described previously (Hammer et al., 1985b). Single-cell fertilized sheep zygotes were collected on d 2 of the estrous cycle. Zygotes were then transferred to sterile tubes containing Medium 199 with Earle's salts, 2.2 g/liter of sodium bicarbonate, and 10% fetal calf serum (pH 7.4). Between 0800 and 0930, zygotes were transported at 39°C from Beltsville, MD to Philadelphia, PA, where they were microinjected with DNA (2 ng/μl; Hammer et al., 1985c). Microinjected zygotes were returned to Beltsville by 1830 and transferred to recipient ewes that had been in estrus at the same time or up to 24 h after the donor ewes. Oviducts of recipient ewes were exposed via mid-ventral laparotomy. Microinjected zygotes were aspirated into a positive displacement glass micropipette in a volume of 5 to 10 μl and expelled 1 to 3 cm into the fimbriated end of the oviduct. In most

cases, one to three zygotes were transferred into the oviducts of each ewe.

Plasma Analysis. Each week, blood samples were collected from lambs between 1000 and 1200. Growth hormone was assayed by RIA (Miller et al., 1989) using HS-X74, anti-bovine GH serum, USDA-bGH-B-1 standard, and USDA-bGH-1 radioiodinated tracer. The crossreactivity with other ovine or bovine pituitary hormones was less than .1%. Recovery of bovine GH from plasma was 100 ± 10%, and the intra- and interassay CV were < 15%. Assay sensitivity was .5 ng/ml. The RIA for GH could not distinguish between native ovine and bovine GH but was used to identify lambs with unusually high or moderate levels of GH. Human GRF was assayed by RIA (Frohman and Downs, 1986). This assay exhibits a 60% crossreactivity with ovine GRF and a sensitivity of 6 pg/ml. For Trf-GH sheep, IGF-I was measured by RIA (Miller et al., 1989) after acidified-ethanol extraction. Recombinant IGF-I[8] was used both as a standard and as the radiolabeled tracer. Anti-human IGF-I serum (CH549/805C) was used as the antibody. Plasma concentrations of IGF-I in the Alb-GRF experiments were assayed by RIA after acidification of plasma samples with glycl-glycine-HCl (Underwood et al., 1982, as modified by Elsasser et al., 1988, 1989). Rabbit anti-human IGF-I (VB 286)[9] was used as the antibody. Recovery of 30, 60, and 120 pg of added IGF-I from plasma averaged 90 ± 4%. The intra-assay CV was 9% and the minimal detectable concentration was 15 pg/tube. Insulin levels were measured by RIA as described by Elsasser et al. (1986). Bovine insulin[10] was used for standards and for radiolabeled tracer. Guinea pig anti-bovine insulin was used as the antibody[11]. The intra-assay CV was 4% and the minimal detectable concentration was 10 pg/tube. Recovery of added hormone from plasma was 95 ± 5%. Beta-hydroxybutyric acid (BOHB) was assayed by enzymatic oxidation of BOHB to acetoacetate with measurement of reduced nicotinamide adenine dinucleotide production at 340 nm using a kit[12]. Plasma and urine were assayed for glucose by the method of Stein (1963) by measuring production at 340 nm.

TABLE 1. PRODUCTION EFFICIENCY OF TRANSGENIC SHEEP

Item	Trf-GH, %[a]	Alb-GRF, %[a]
Ova injected	247	171
Offspring	42 (17.0)	16 (9.4)
Transgenic	11 (26.2)	4 (25.0)
Expressing	3 (27.3)	2 (50.0)

[a]Trf-GH = transferrin-growth hormone, Alb-GRF = albumin-growth hormone-releasing factor. Percentage of preceding number in column.

DNA and RNA Analysis. Integration of microinjected DNA into lambs was determined by dot hybridization of DNA prepared from tails (Brinster et al., 1985). The labeled probes were previously described (Hammer et al., 1985a,b). Bovine growth hormone RNA was measured by solution hybridization with a ^{32}P-labeled oligonucleotide (21-mer) (Ornitz et al., 1985). Human growth hormone-releasing factor RNA was analyzed by Northern blot.

Feed Studies. In the Trf-GH experiments, lambs were penned singly and feed intake was determined daily for 90 d beginning at 68.8 ± 1.4 d of age. Lambs were weighed weekly. In the Alb-GRF experiments, lambs were fed in the same way except that feed intake was measured for only 63 d. The diet was a pelleted mix of corn, soybean meal, cracked barley, orchard grass hay, and minerals calculated to have 13.9% CP and 2.73 Mcal of ME/kg.

Results

Production of Transgenic Sheep. Forty-two sheep (17%) were obtained from 247 zygotes microinjected with the Trf-GH gene construct (Table 1). Eleven (26%) of these carried the Trf-GH transgene. Three (27%) of the 11 expressed the transgene. These were ram 8727, ewe 8741, and fetus 770-2. Sixteen (9%) lambs were born from 171 eggs microinjected with the Alb-GRF fusion gene. Four (25%) of these carried the Alb-GRF transgene. Two (50%) of the four expressed the transgene. These were ewe 8824 and ram 8769. Lambs

[8]Amgen, Thousand Oaks, CA.
[9]National Hormone and Pituitary Agency, Baltimore, MD.
[10]Novo Research, Copenhagen, Denmark.
[11]Cappel, King Of Prussia, PA.
[12]Sigma Chemical Co., St. Louis, MO.

TABLE 2. HUMAN GROWTH HORMONE-RELEASING FACTOR (GRF) LEVELS IN PLASMA AND TISSUE FROM ALBUMIN-GRF SHEEP

Lamb sex	Tissue	GRF, pg/ml
8824 Ewe	Plasma pool at 1 to 2 mo	683.0
	Plasma at necropsy	51.0
8769 Ram	Plasma pool at 1 to 2 mo	264.0
Controls[a]	Plasma pool at 1 to 2 mo	10 to 30

[a]N = 5.

8727, 8741, and fetus 770-2 that expressed Trf-GH had 1, <1, and <1 copy of the transgene, respectively. Lambs 8769 and 8824 that expressed Alb-GRF had 10 copies and 1 copy of the transgene, respectively. The nonexpressing Trf-GH lambs all had two or fewer copies of the transgene in their genome.

Circulating Levels of Growth-Related Peptides. Plasma pooled between 1 to 2 mo of age from ewe 8824 and ram 8769 contained 683 and 264 pg/ml of human GRF, respectively (Table 2). Levels of GRF in all other control and nonexpressing transgenic lambs were between 10 and 30 pg/ml.

Growth hormone levels from ram 8769, ewe 8824, and paired controls are presented in Figure 2A. Chronically elevated levels of endogenous ovine GH, ranging from approximately 30 to 50 ng/ml, were observed between 4 and 21 wk of age. After 21 wk of age (near the time of death), GH levels decreased to control values. Insulin-like growth factor I levels were also assayed in plasma from ram 8769 and ewe 8824 (Figure 2B). Elevated levels of IGF-I were not consistently observed in these sheep.

Similar measurements were performed on Trf-GH expressing sheep. Growth hormone chronically elevated in both ram 8727 (~100 ng/ml) and ewe 8741 (~200 ng/ml) (Figure 2A). Levels of IGF-I were also elevated in plasma for both expressing Trf-GH sheep. Ewe 8741 had circulating levels of IGF-I at approximately 500 ng/ml, and ram 8727 had levels that continuously increased from 100 up to 1,200 ng/ml (Figure 2). Growth hormone and IGF-I values in control and nonexpressing sheep were approximately 5 ng/ml and 200 ng/ml, respectively.

Transgene Expression. Steady-state levels of transgene RNA in various tissues of Trf-GH transgenic sheep were measured by solution hybridization (Table 3). Variable levels of bGH-RNA were found in a variety of tissues in the Trf-GH sheep. Relatively high levels of bGH-RNA were found in the liver and heart of ewe 8741, and high levels of bovine GH RNA were found in the gut of fetus 770-2. Ram 8727 expressed transgene RNA predominantly in the brain and spleen. Human GRF RNA was found in all tissues analyzed; highest levels were found in the liver and pituitary of Alb-GRF ewe 8824 by Northern blot analysis (Figure 3).

Growth of Transgenic Sheep. The growth of Trf-GH expressing transgenic ram 8727 for the first 21 wk of life was similar to that observed in a paired control and in contemporary control lambs (data not shown; Rexroad et al., 1988). About the same amount of feed for each kilogram of growth was required by ram 8727 and the paired control (Table 4). In contrast, the Trf-GH expressing ewe 8741 grew at a nearly normal rate until about 15 wk of age, after which she grew less rapidly than her paired control. Lamb 8741 required almost twice as much feed for each kilogram of growth (Table 4).

Transgenic ewe 8824 expressing Alb-GRF grew at a nearly normal rate in comparison to her paired control and contemporary controls (data not shown), with approximately normal growth efficiency for the first 17 wk of life. Transgenic ram 8769 expressing Alb-GRF was small at birth and failed to exceed 12 kg in weight.

Pathology Associated with Transgenic Sheep. Transferrin-growth hormone ram 8727 seemed to develop normally. This animal lived to approximately 7.5 mo of age and failed to

TABLE 3. TRANSGENE mRNA LEVELS IN TISSUES FROM TRANSFERRIN-GROWTH HORMONE SHEEP[a]

	Sheep (sex)		
Tissue	770-2 (Ewe)	8727 (Ram)	8741 (Ewe)
Heart	12.0	0	43.8
Liver	2.0	0	36.1
Gut	40.0	0	0
Brain	0	6.9	8.2
Kidney	12.0	0	2.9
Spleen	0	13.3	2.8
Lung	0	0	0
Pituitary	0	0	0
Muscle	4.0	0	0

[a]Values are picograms of bovine growth hormone RNA/μg of total nucleic acid.

mature sexually. His testicles were small and almost no sperm could be found in the epididymides. Expressing Alb-GRF ewe 8724 seemed to have severe bowing of the front legs. Although the condition is unusual, it was observed in a nonexpressing Trf-GH transgenic ram (8748) and may reflect a genetic characteristics of the flock used to generate these lambs. Both ewes 8741 and 8824 died at about 6 mo of age and had reproductive tracts that were prepuberal in appearance. Neither of the ewes had ovulated.

All three transgenic lambs with elevated GH levels lost weight immediately before death. At necropsy the lambs were found to have little subcutaneous fat and almost no perirenal fat. Loss of body fat or failure to deposit fat was probably related to the diabetes-like condition observed in these animals.

Development of Diabetes. Plasma glucose levels were chronically elevated after 14 wk of age in both types of expressing transgenic sheep (Figure 4). Glucose levels up to approximately 400 mg/dl were observed in both expressing Trf-GH sheep and up to 300 mg/dl in Alb-GRF ewe 8824, compared with levels in control animals of approximately 50 mg/dl.

Plasma insulin levels in Trf-GH and Alb-GRF sheep also became elevated. Insulin

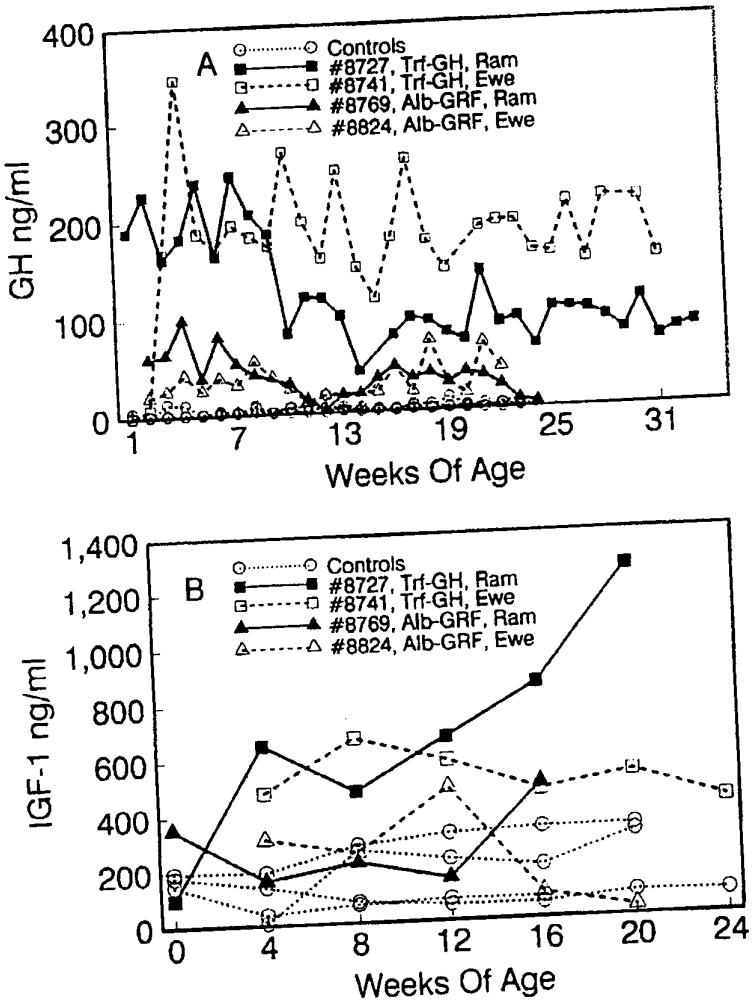

Figure 2. Panel A presents plasma growth hormone (GH) in transferrin-GH (Trf-GH) and albumin-growth hormone-releasing factor (Alb-GRF) transgenic lambs as determined by radioimmunoassay of weekly samples. Panel B presents plasma insulin-like growth factor (IGF-I) as determined by radioimmunoassay of monthly samples.

TABLE 4. GROWTH EFFICIENCIES OF TRANSGENIC SHEEP[a]

Lamb	Sex	Gene	ADG	Feed/gain
8725	Ram	Control	.292	4.18
8727	Ram	Trf-GH	.257	4.84
8736	Ewe	Control	.194	4.67
8741	Ewe	Trf-GH	.133	8.08
8827	Ewe	Control	.317	4.84
8824	Ewe	Alb-GRF	.270	4.63

[a]Transferrin-growth hormone (Trf-GH) sheep on feeding study for 84 to 93 d; albumin-growth hormone releasing factor (Alb-GRF) sheep on feeding study for 63 d.

levels up to 10 and 16 ng/ml for Trf-GH ram 8741 and ewe 8727, respectively, and 7 ng/ml for Alb-GRF ewe 8824 were observed (Figure 4). These elevated levels of insulin decreased before death. Control insulin levels were 1 to 3 ng/ml.

Plasma BOHB concentrations were elevated in the expressing transgenic lambs, paralleling the high glucose levels found after 14 wk of age, which indicated that the animals were ketotic (not shown).

Discussion

In previous reports the efficiency of producing transgenic sheep (transgenic lambs/zygotes injected) has ranged from .1% (Hammer et al., 1985c) to 2.07% (Rexroad et al., 1989). The

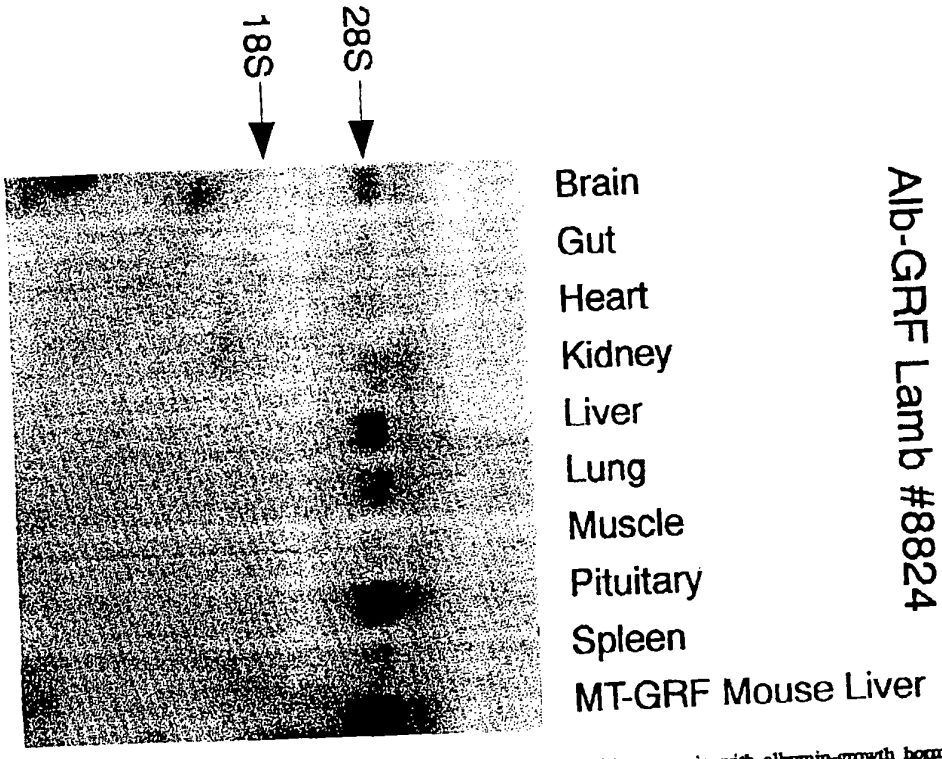

Figure 3. Northern blot of RNA isolated from tissues of lamb #8824 transgenic with albumin-growth hormone-releasing factor (Alb-GRF). Most tissues contained detectable RNA but liver and pituitary revealed the highest levels. The right-hand lane is RNA from a mouse transgenic with metallothionein-I-GRF (MT-GRF) that had a high level of GRF RNA in the liver. Growth hormone-releasing factor RNA was not seen in tissues of nonexpressors or controls.

efficiency of production of transgenic sheep for the Trf-GH (4.45%) and Alb-GRF (2.34%) genes in this study was similar to that found in our most recent study for an MT-GRF (2.07%) gene (Rexroad et al., 1989). Two gene constructs consisting of the ovine β-lactoglobulin promoter ligated to either the gene for human blood clotting factor IX or the human α-1 antitrypsin produced transgenic sheep with efficiencies of 1.30 and 2.04%. In both cases high viability (> 18% zygotes producing lambs) of the microinjected zygotes was observed (Simons et al., 1988). In other studies with lower production efficiencies, viability of manipulated sheep zygotes was < 8% (Hammer et al., 1985c; Pursel et al., 1987; Ward et al., 1989). Viability of zygotes microinjected in this study with the Trf-GH gene construct was high (17.1%). A major source of reduced viability of zygotes resulting

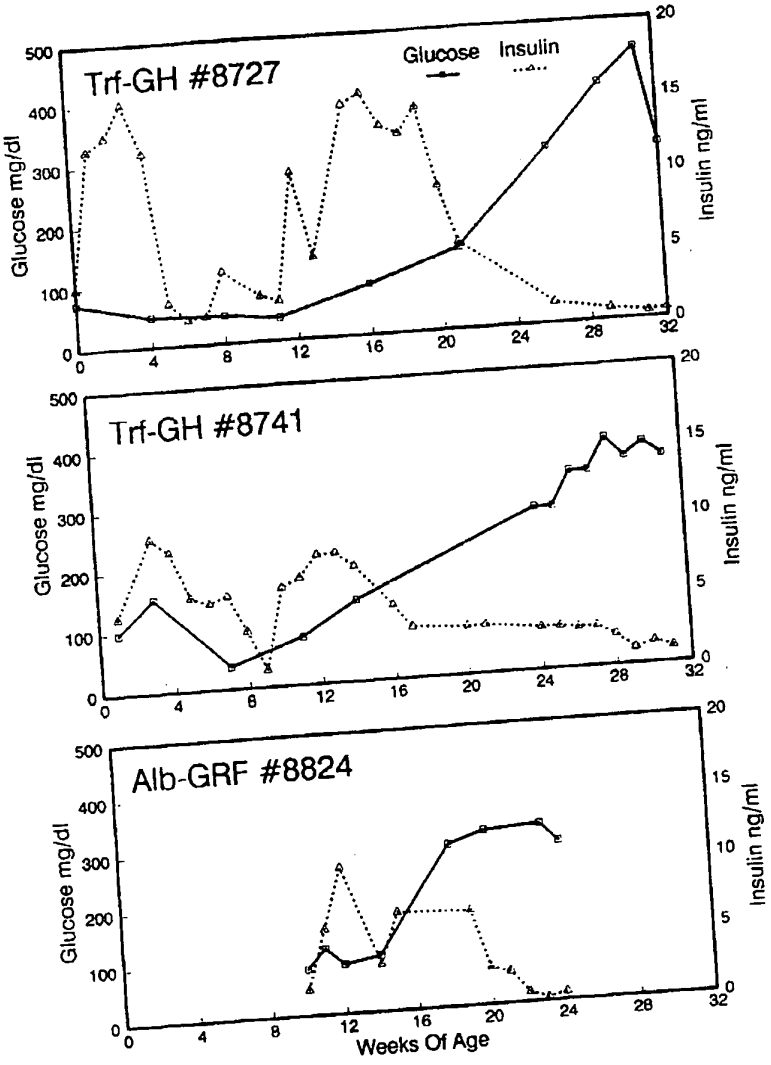

Figure 4. Each panel presents glucose and insulin values from a single transgenic lamb. For clarity, control values are not shown. The mean (± SEM) low glucose value for controls (n = 8) was 32 ± 3.6 mg/dl, and the mean high value was 68 ± 6.4 mg/dl sampled over the same interval and at the same frequency as in transgenic lambs. The highest glucose in any single control sample was 111 mg/dl. The mean (± SEM) low insulin value for controls (n = 8) was .5 ± .11 ng/ml, and the mean high value was 4.2 ± .69 ng/ml sampled over the same interval and at the same frequency as in transgenic lambs. The highest insulin value in any single control sample was 7.8 ng/ml.

from the microinjection procedure is injection of buffer and(or) DNA into a pronucleus (Rexroad and Wall, 1987). Refined microinjection procedures may account for the higher viability and integration rates currently observed.

The Trf-GH gene was active in sheep, producing elevated levels of bovine GH in plasma. In mice, the transferrin gene is expressed at high levels, predominantly in liver, and at lower levels in brain. This pattern of expression was observed in transgenic mice carrying a fusion gene composed of the mouse Trf enhancer/promoter ligated to a human GH structural gene (Idzerda et al., 1989). However, no consistent tissue-specific pattern of expression was observed for the Trf-GH gene in the three transgenic sheep. Expression was high in liver and heart in one animal, and high levels of bovine GH RNA were found in gut in another. Expression was undetectable in liver of ram 8727. Thus, although the mouse Trf enhancer/promoter seems to be active in sheep, proper regulation was not observed. It is possible that the factors required for tissue-specific transcriptional activation of the mouse Trf gene are not present in ovine cells or that ovine factors do not interact efficiently with the mouse Trf regulatory region. Transgenic sheep carrying a fusion gene composed of the ovine metallothionein promoter ligated to the ovine GH gene expressed GH at levels greater than those observed using the mouse MT promoter, also suggesting species-specific effects on expression of foreign genes (Ward et al., 1989). Such species considerations may be important in selection of the regulatory regions for specific genes used in generating transgenic livestock. Alternatively, the variation observed could be explained by influences specific to the site of transgene integration.

The Alb-GRF gene was also active in sheep. In mice, the Alb gene is expressed specifically in liver, and transgenic mice harboring Alb fusion genes generally follow this tissue-specific pattern of expression (Pinkert et al., 1987; Sandgren et al., 1989). This pattern was not observed in the Alb-GRF ewe 8824, although expression in liver was relatively high. Thus, the mouse Alb enhancer/promoter is not functioning properly in this transgenic sheep. The lack of expression in liver may be due to regulatory elements residing within the human GRF sequences. Alternatively, the combination of Alb and GRF sequences may have created a novel element specifying ubiquitous transcriptional activity (Swanson et al., 1985).

The activity of the mouse Trf promoter in sheep resulted in the ectopic production of GH, leading to the induction of elevated levels of endogenous IGF-I. Similarly, the mouse Alb promoter/enhancer redirected expression of GRF to many tissues, which in turn induced elevated levels of endogenous sheep GH. These results demonstrate that the expression of foreign growth-related genes in sheep is capable of stimulating the cascade of endogenous hormones associated with growth. Despite the elevated levels of GH and IGF-I, both of which have central roles in growth, these sheep failed to grow faster than controls. Furthermore, these expressing transgenic sheep, unlike pigs with elevated GH, did not demonstrate enhanced feed efficiency. However, similar to pigs with elevated GH, there did seem to be a significant and sizable decrease in fat deposits (e.g., in subcutaneous and perirenal areas; Pursel et al., 1989).

Pathologies were found in all of the transgenic sheep that expressed the transgenes. Indeed, all died at a relatively early age, whereas nonexpressing transgenic sheep carrying the same genes were unaffected. Most prominent among these pathologies was a diabetic condition that developed in both types of expressing transgenic sheep. Glucose and insulin levels became chronically elevated. Before death, insulin levels dropped and BOHB levels increased, indicating ketosis. The utilization of fat as an energy source would explain the lack of fat observed in the sheep at autopsy and is consistent with the development of ketone bodies associated with inadequate intake of utilization of ME. Transgenic pigs chronically expressing foreign GH also develop a variety of pathologies (Ebert et al., 1988; Pursel et al., 1989), many of which are also prevalent in general swine populations. The observations of Pursel et al. (1989) suggest that the severity and frequency of these conditions may be increased by chronic exposure to elevated levels of foreign GH.

Transgenic mice chronically expressing foreign GH or GRF that grow at accelerated rates compared with controls exhibit hepatomegaly and glomerulosclerosis (Doi et al., 1988; Quaife et al., 1989). These animals die earlier than controls, perhaps because of the kidney lesions induced by chronic exposure to GH.

Chronically elevated insulin levels were observed in these mice, but the animals were normoglycemic. It is possible that they succumb to the kidney lesions before the development of the diabetic condition observed in our transgenic sheep. Alternatively, species differences may account for the differences observed among sheep, pigs, and mice. Mice seem to be most responsive to growth enhancement. Sheep, on the other hand, are apparently least responsive to growth induction and are more likely to develop a GH-induced diabetic syndrome. Such species differences make the beneficial use of growth-related genes a complicated problem, perhaps resolvable only through the use of alternative gene regulatory control elements or entering into the growth process through other mechanisms (e.g., local growth regulators). For example, transgenic mice chronically expressing human IGF-I grow approximately 30% larger than controls with selective organomegaly (Mathews et al., 1988), yet they are relatively free of pathology (Quaife et al., 1989). These results suggest that chronic expression of IGF-I in sheep may be a strategy for increasing the production efficiency in this species without the pathologies associated with excess GH.

Implications

The results of this research broaden the number of promoters/enhancers elements that can be used to produce transgenic sheep that express foreign genes to include the mouse transferrin promoter and the mouse albumin promoter/enhancer. Mouse promoters/enhancers, however, may not always maintain tissue-specific expression in sheep. The findings demonstrate that lambs are extremely sensitive to chronically elevated foreign or endogenous growth hormone and are susceptible to a fatal diabetes. The utility of the transgenic approach to enhancing growth hormone secretion to improve growth is limited by our ability to insert functional transgenes that can be regulated at an appropriate level of expression.

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APPENDIX K

TRANSGENIC PRODUCTION OF A VARIANT OF HUMAN TISSUE-TYPE PLASMINOGEN ACTIVATOR IN GOAT MILK: GENERATION OF TRANSGENIC GOATS AND ANALYSIS OF EXPRESSION

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We report the first successful production of transgenic goats that express a heterologous protein in their milk. The production of a glycosylation variant of human tPA (LAtPA - longer acting tissue plasminogen activator) from an expression vector containing the murine whey acid promoter (WAP) operatively linked to the cDNA of a modified version of human tPA was examined in transgenic dairy goats. Two transgenic goats were identified from 29 animals born. The first animal, a female, was mated and allowed to carry the pregnancy to term. Milk was obtained upon parturition and was shown to contain enzymatically active LAtPA at a concentration of 3 μg/ml.

Several types of human proteins have been expressed in lactating mammary glands of transgenic mice and the proteins secreted into milk (reviewed in refs. 1,2). The scale-up of mouse model systems to livestock species may ultimately provide an alternative production system to the commonly used mammalian tissue culture production processes. A transgenic mammary gland production system would have the potential advantages of not requiring an intensive capital expenditure in setting up a manufacturing facility, and of providing a highly cost efficient system due to high expression levels and low expendable production costs.

Most of the work to date has been done in the mouse model system. Regulatory sequences of the whey acid protein (WAP), β- and α-lactoglobin (BLG) and β-casein genes have been used to target expression of numerous genes to the lactating mammary gland, including tissue plasminogen activator (tPA)[3,4], human anti-hemophilic factor IX[5], soluble CD4[6], human interleukin-2[7], and human α_1-antitrypsin[8]. A vector system based on the αS1-casein gene has also been tested in a mouse model where human urokinase was produced efficiently in milk at levels 10 times greater than those achieved in cellular expression systems[9].

Whereas model systems have demonstrated the feasibility of targeting gene expression to the lactating mammary gland and secretion of heterologous proteins into milk, efficient generation of transgenic livestock and production of foreign proteins in their milk have proven more difficult to achieve. Early work aimed at the generation of transgenic farm animals led to low frequencies of integration, low number of animals that expressed the recombinant proteins, reproductive problems, and resultant physiological problems[2]. By targeting protein synthesis to an exocrine organ in which expressed proteins would be expected to be sequestered away from the circulation and removed from the animal, we and others hope to bypass some of these potential problems in the generation of transgenic livestock. In fact, transgenic sheep, which produce factor IX in their milk, have been generated and apparently exhibit no physiological or reproductive problems[5], although expression levels in these animals were low. More recently, however, high expression of the murine whey acidic protein in transgenic swine and mice may have had adverse effects on the physiology of the mammary gland[10].

We have aimed to produce a commercial prototype for the large-scale manufacture of high market-volume proteins in the transgenic mammary gland system using the dairy goat as a production animal. The goat was chosen for several reasons: (1) Dairy goats produce large volumes of milk, on average 4 liters per day; (2) goats have gestation and development periods of moderate length (5 and 8 months respectively); and (3) goat milk has been extensively characterized at the biochemical level[11]. In this paper, we describe the first successful generation of transgenic goats at frequencies that approach those in the rodent systems. More importantly, a transgenic goat was generated that produced an enzymatically active form of tPA throughout a normal lactation period. These experiments further support the concept of targeting expression of transgenes that encode pharmaceutical proteins to the mammary gland of dairy livestock.

RESULTS AND DISCUSSION

Generation of transgenic goats. The expression vector WAP-tPA was generated previously by fusing a 2·6 kb EcoRI-KpnI fragment upstream of the murine whey acid protein gene to a cDNA encoding wild type human tPA[3]. This vector led to expression of tPA in milk of transgenic mice at levels as high as 250 μg/ml (data not shown). A structural tPA variant was constructed (designated LAtPA) in which an asparagine to glutamine point mutation was introduced into the cDNA to produce a recombinant protein devoid of glycosylation at residue Asn 117. This longer acting tPA variant had an increased systemic half-life in a rabbit model[12]. A DNA fragment containing this point mutation in the tPA cDNA was substituted for the equivalent fragment in WAP-tPA to generate the vector used in this study, WAP-LAtPA (Fig. 1).

Goat embryos were flushed surgically from the oviducts of superovulated dairy goats as described in the Experimental Protocol. The superovulation protocol had been

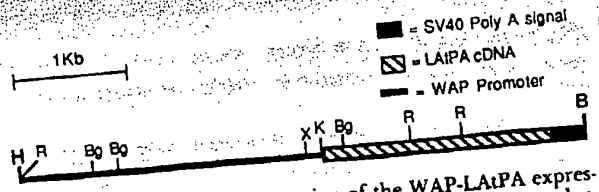

FIGURE 1 A schematic representation of the WAP-LAtPA expression vector and its restriction enzyme sites used for the production of transgenic goats. H = HindIII, R = EcoRI, Bg = BglII, X = XbaI, B = BamHI, K = KpnI.

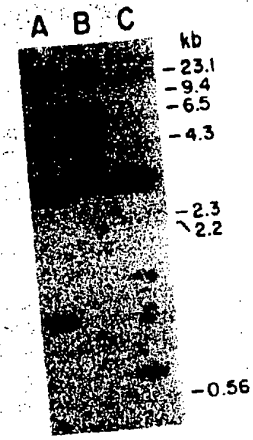

FIGURE 2 Southern blot hybridization of transgenic goat DNA. DNA isolated from the blood of goat #1 was digested, fractionated on an agarose gel, and hybridized to a probe from the whey acid protein gene as described in the Experimental Protocol. Hybridization of this probe to DNA from non-transgenic animals gave no signal (data not shown). Lanes A: BglII; B: XbaI; C: EcoRI.

previously optimized to result in the highest yield and proportion of one-cell embryos[13]. In the course of this study, a total of 372 embryos or ova were collected from 63 donor animals. Of the embryos collected, 252 (68%) were zygotes, 24 (7%) were 2-cells, 5 (1%) were 4-cells and 91 (25%) were unfertilized as determined by the absence of pronuclei. Twenty-eight percent of the injected fertilized embryos were considered poor injections due to the non-optimal positioning of the injected pronuclei, i.e., the pronuclei were at the periphery of the cell making the nuclear injection very difficult. Approximately 8% of the fertilized embryos collected had to be centrifuged for 30 seconds at 13,000 × g to visualize the pronuclei under Normarski optics. Due to the limited number of recipients and donors available on any given experimental day, microinjected 1-cell, centrifuged 1-cell, and 2-cell goat embryos were typically mixed prior to transfer to the recipient females. This prevented us from confirming the viability of the centrifuged eggs or the potential production of transgenic goats by microinjection of 2-cell embryos. However, subsequent experiments with other fusion genes have confirmed that microinjected centrifuged 1-cell goat embryos are viable and can produce viable offspring. In addition, we have also produced a transgenic goat (potentially a mosaic) that was the result of injection of a 2-cell goat embryo (data not shown).

Embryos were injected with a 4.9 kb HindIII-BamHI fragment of WAP-LAtPA purified free of procaryotic DNA (Fig. 1) at a concentration of 1 μg/ml in 10 mM Tris, pH 7.5, 0.1 mM EDTA, and either immediately transferred to the oviducts of recipient females or cultured in Ham's F12 medium containing 10% fetal calf serum in an atmosphere of 5% CO_2 in air at 37°C for 72 hours and subsequently transferred to the uterus of recipient females. Typically the cultured goat embryos were blocked at the 8-16 cell stage in this culture system, but remained viable and could produce live offspring (Table 1).

Pregnancies were confirmed by the inability of recipient animals to return to natural estrus and by ultrasonic examination on days 45 and 55 of pregnancy. Ultrasound on days 45 and 55 of pregnancy have resulted in a 100% early pregnancy confirmation over the first three years of this project. A complete summary of the recipient data is shown in Table 1. Twenty-nine animals were born from 203 embryos transferred, representing 14% of injected embryos surviving to term. Samples of blood and ear tissue from each goat were analyzed by Southern blotting in order to detect transgenics and to confirm that mosaic animals did not escape detection. Two animals were identified as transgenic; a female, goat #1, and a male, goat #21. The generation of 2 transgenic animals in 29 goats born from injected embryos represents an integration rate of 7% and can be compared to frequencies previously reported for mouse (10–30%[14]), rabbit (9.5%[7]), pig (10–40%[15]) and sheep (6–10%[16,5]).

At nine months of age, goat #1 was mated to a non-transgenic male. She became pregnant without difficulty and delivered two non-transgenic progeny. A second pregnancy resulted in three additional offspring, one of which was transgenic. Goat #21 (male) was aspermic and was subsequently diagnosed to have a bilateral spermatocele at the head of the epididymis resulting in blockage of normal, developed sperm from entering the vas deferens.

TABLE 1 Summary of injected goat embryos transferred to recipient females.

A.

No. Recipients	Centrifugation	Transfer To	No. Embryos/Stage	No. Pregnant	No. Offspring	No. Transgenic
28	No	Oviduct	137/1-cell	13[a]	22	2
			18/2-cell	0[a]	0	0
2	Yes	Oviduct	10/1-cell	4	5	0
5	No	Uterus	31/morula[b]	1	2	
1	Yes	Uterus	7/morula[b]			

B. Efficiency

No. Recipients	No. Embryos	No. Pregnant (%)	No. Offspring (%)	No. Transgenic (%)
36	203	18 (50.0)[c]	29 (14.3)[d]	2 (6.9)[c]

[a]Two of the pregnant animals aborted prematurely.
[b]Embryos injected at the 1-cell stage and cultured for 72 hours.
[c]Percentage is the number of pregnant animals per number of recipients.

probable congenital defect that is commonly seen in goats. Therefore, we are unclear as to whether this defect relates to the integration of the transgene.

In order to determine the arrangement of the transgene and to estimate the number of copies per cell, blood DNA from the founder female (#1) was digested with three endonucleases and analyzed by Southern blot (Fig. 2). The blot was probed with a 580 bp EcoRI-BglII fragment from the 5′ end of the WAP upstream region labeled to a specific activity of 1×10^8 cpm/μg of DNA by the random hexamer labeling technique[17]. BglII digestion (Lane A) resulted in a predictable 2.9 kb fragment and a 5′ junction fragment approximately 1.0 kb. BglII is known to cut at three sites within the fusion gene (Fig. 1). The lower intensity of the junction fragment indicates that the integrant was inserted at one site and had multiple copies. XbaI (Lane B) cuts once within the fusion gene resulting in a predictable 4.9 kb fragment if the fusion gene integrated in multiple copies and in a tandem array in normal orientation, as well as 5′ junction fragment of approximately 3.3 kb. EcoRI cuts the fusion gene into three fragments (Fig. 1) of which the 3.3 kb fragment will hybridize to the probe on the Southern blot (Lane C). Figure 3 shows a Southern blot of an EcoRI digest of DNA extracted from blood cells from goat #1 and her five offspring. The blot was probed with a 1.7 kb LAtPA cDNA fragment. The Southern blot shows 3 major bands representing 3.3 kb, 1.1 kb and 472 bp fragments that corresponds to the 5′, 3′, and interior components of the fusion gene respectively. A minor band of 1.6 kb represents the 3′ junction fragment. The probe is weakly homologous to goat DNA. The initial restriction pattern of genomic DNA from the founder female was consistent with the presence of one to two integrated copies of the tPA transgene. However, as shown in Figure 3, the transgenic offspring was shown to contain more copies of the transgene per cell than the founder. This can be interpreted to mean that the founder animal was a mosaic with more copies of the fusion gene per cell than was originally estimated. Additionally, when the blots were counted on a Beta Scope, the data indicated that the bands in offspring 1-3 were twice the value of the #1 founder and supports the concept that the female founder was a mosaic (data not shown). It is estimated from the Southern analysis that the transgenic offspring contained approximately 3-5 copies of the transgene per cell. It should be noted that Southern analysis of DNA from ear tissue gave identical information.

Expression of tPA in milk. The transgenic mother was milked manually twice per day with an average daily yield of 3-4 liters. Milk was stored frozen at −20°C and thawed just prior to analyses. ELISA and amidolytic assays were run on representative milk samples from the first two months of lactation with continued ELISA assay up to the end of lactation (240 days). The daily volume and concentration of LAtPA is shown in Figure 4A. The animal expressed LAtPA at approximately 3 μg/ml during the peak lactation period (1 to 140 days) with an increase in concentration (6.0 μg/ml) toward the end of the lactation period (141 to 240 days). The second lactation produced LAtPA at the same concentration as the first lactation. The first lactation period was truncated from a normal 300 days to 240 days to eliminate the hand milking procedure. The daily milk output and lactation curve were characteristic of a normal dairy goat during her first lactation. The apparent rise in output of LAtPA during the later part of the lactation period did not parallel the constant total protein concentration in the same milk samples. The elevated milk production from September 17 to November 12 corresponds to the initiation of her second estrus season (first

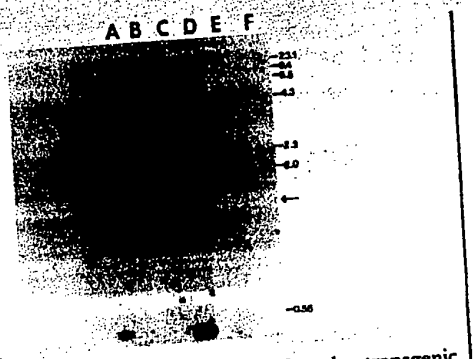

FIGURE 3 Southern blot hybridization of the founder transgenic goat #1 (Lane A) and her five kids (Lanes B, C, D, E, F). DNA isolated from blood was digested with EcoRI, fractionated on an agarose gel, and hybridized to a 1.7 kb LAtPA cDNA fragment. Hybridization of this probe to the transgene shows 3 bands: A: 3.3 kb 5′ junction fragment (Band 1); a 1.1 kb 3′ fragment (Band 2); a 472 bp interior fragment (Band 3). A minor band of 1.6 kb represents the 3′ junction fragment (arrow). Kid #1-3 (Lane D) was shown to be transgenic. Note the intensity of the bands from kid #1-3 was twice as intense as the founder female #1 and indicates that the founder animal is probably a mosaic.

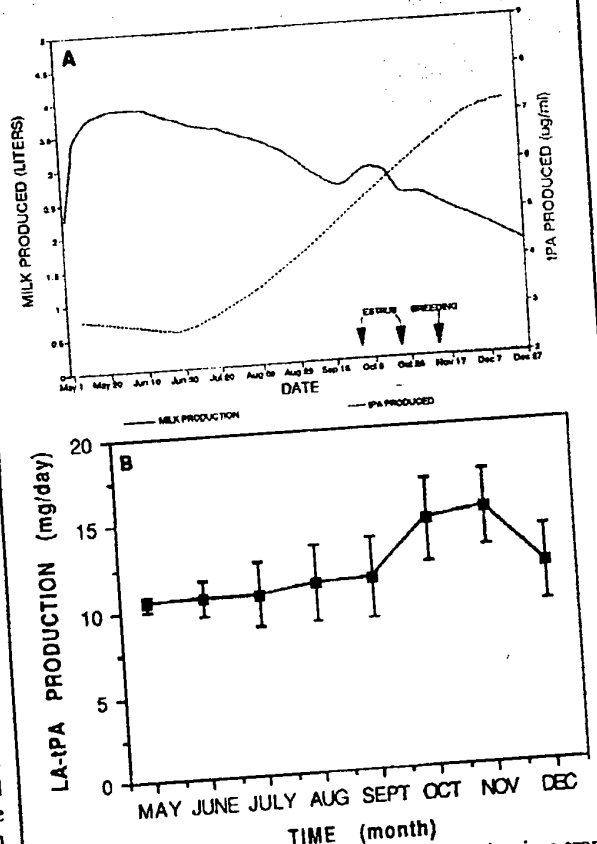

FIGURE 4 (A) LAtPA production throughout lactation in a transgenic goat. The solid line represents the best fit line for daily milk production throughout the 240 day lactation period. The dotted line represents the best fit line for the daily concentration of LAtPA in the milk. The days of her first, second and third estrus cycle and breeding are indicated on the graph. (B) Stability of expression of LAtPA in the milk of a transgenic goat (#1). Milk was harvested and stored as described in the accompanying paper. The concentration of the recombinant enzyme in the milk was determined by ELISA. Total LAtPA produced per day was calculated and daily averages and standard deviations for individual months determined by pooling the data from seven days during the course of each month.

estrus = October 3; second estrus = October 24; third estrus = November 12). The total amount of LAtPA produced per day was calculated and daily averages and standard deviations for individual months are shown in Figure 4B. The output of LAtPA remained relatively constant during the course of lactation, ranging between 11 and 15 mg/day. Although the concentration of the enzyme in milk was relatively low, i.e., approximately 10% of that observed in the recombinant C127 cell line, the continual expression was encouraging since it implied consistent production can be achieved in transgenic animals. The slight rise in output of LAtPA during October and November corresponds to estrus activity. Although the basis for this increase is not known, it is possible that the hormonal changes in the animal associated with the breeding season could have some effect on the expression of the transgene. The female was successfully rebred on November 12 and 13. Interestingly, this period of estrus cyclicity corresponded to a significant rise in the total amount of LAtPA being produced per day. The LAtPA produced in the milk was enzymatically active at approximately 610,000 U/mg. A detailed characterization of the protein is reported in an accompanying paper (Denman et al., *Bio/Technology: This issue*).

These experiments show that targeting transgenes that code for medically important pharmaceutical proteins to the mammary gland of dairy goats is feasible. The level of LAtPA was not high (3 μg/ml) in this first transgenic goat. The likelihood that this goat is mosaic may not allow us to achieve the actual expression level of this gene construct until we generate an F_1 female. However, we have recently produced another female transgenic goat that is producing LAtPA from a β-casein promoter at 2-3 mg/ml (data not shown). At this concentration, the dairy goat may be an economically viable bioreactor for human pharmaceuticals.

EXPERIMENTAL PROTOCOL

Production of transgenic goats. Goats used as donor animals were of either Alpine or Saanen breeds. The timing of estrus was synchronized in the donors with norgestomet ear implants (Syncromate-B, CEVA Laboratories, Inc., Overland Park, KS; 6 mg). Prostaglandin was administered after the first 7-9 days to remove endogenous sources of progesterone. At day 13 following progesterone administration, follicle-stimulating hormone (FSH, Schering Corp., Kenilworth, NJ) was given to goats at a dose of 18 mg over three days in twice daily injections (Warren Foote, personal communication). During the anestrus season (after February), the dose of FSH was increased to 24 mg administered similarly over three days in twice daily injections. Twenty-four hours following implant removal, the donor animals were mated several times to fertile males over a two-day period. Recipient animals were synchronized by the same protocols as the donor animals except that a single non-superovulatory injection of pregnant mares serum gonadotropin (PMSG, Sigma, St. Louis, MO) was given on day 13 of progesterone treatment in place of the FSH. From September to January, the recipients received 400 IU PMSG, and from February to April they received 750 IU PMSG. Recipient females were mated to vasectomized males to ensure estrus synchrony. Seventy-two hours following implant removal, embryos were recovered surgically from the oviducts of donors. Embryos were flushed from oviducts associated with ovulated ovaries through a cannula with sterile phosphate-buffered saline and were collected in a petri dish as previously reported[15]. The HindIII-BamHI fragment of WAP-LAtPA was injected into one of the two pronuclei from one-cell embryos or into a nucleus of one blastomere of two-cell embryos at a concentration of 1 μg/ml. Embryos were surgically transferred into the oviducts of the recipient females or to the uteri following a 72 hour culture period.

Identification of transgenic goats. DNA was extracted from the buffy coat recovered from blood of goat #1. Following digestion with restriction enzymes as indicated in the legend to Figure 1, DNA was fractionated and blotted onto nitrocellulose[18]. The probe was a 1.7 kb LAtPA cDNA isolated from the region of the whey acid protein gene 2600 bp upstream of the transcriptional start site[3]. The probe was radioactively labeled by the random primer method[17].

Enzyme activity and protein assays. Plasminogen activator concentrations (amidolytic activity) were determined with an indirect method using the plasmin substrate Val-Leu-Lys-*p*-nitroanilide[12] (S-2251, Helena Labs, Inc.). LAtPA concentration was estimated using the Imubind® tPA ELISA assay kit (American Diagnostics, Chicago, IL) adapted to determine LAtPA in goat's milk.

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Attachment A

APPLICANT FACSIMILE OF FORM PTO-1449 REV 7-80 / U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTY DOCKET NO.	SERIAL NO.
LIST OF PUBLICATIONS CITED BY APPLICANT (Use several sheets if necessary)	BBI-009C4CN	09/241,347
	APPLICANT: Hermann Bujard and Manfred Gossen	
	FILING DATE: February 2, 1995	GROUP: 1632

U.S. PATENT DOCUMENTS

EXAMINER INITIAL		DOCUMENT NUMBER	DATE	NAME	CLASS	SUBCLASS	FILING DATE IF APPROPRIATE
	AA	5,221,778	6/93	Byrne et al.	800	2	
	AB	4,833,080	05/89	Brent et al.	435	172.3	

FOREIGN PATENT DOCUMENTS

		DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES	TRANSLATION NO
	AC	WO 94/04672	03/94	PCT				
	AD	WO 92/20808	11/92	PCT				
	AE	WO 91/19784	12/91	PCT				
	AF	WO 93/04169	03/93	PCT				
	AG	WO 91/19796	12/91	PCT				
	AH	WO 92/11874	07/92	PCT				
	AI	EP 0 332 416	09/89	EPO				
	AJ	WO 93/23431	11/93	PCT				
	AK	WO 94/18317	08/94	PCT				
	AL	0 455 687 B1	11/91	EPO				
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	AN	0 494 724 A2	07/92	EPO				

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	AO		Hinrichs, W., et al., (1994) "Structure of the Tet Repressor-Tetracycline Complex and Regulation of Antibiotic Resistance", *Science*, Vol. 264, pp. 418-420;
	AO'		Hecht, B., et al., (1993) "Noninducible Tet Repressor Mutations Map from the Operator Motif to the C Terminus", *Journal of Bacteriology*, Vol. 175, No. 4;
	AP		Gossen, M., et al., (1993) "Control of gene activity in higher eukaryotic cells by prokaryotic regulatory elements", *TIBS*, Vol. 18, No. 12, pp. 471-475;
	AQ		Fieck, A., et al., (1992) "Modification of the *E. Coli* Lac Repressor for Expression in Eukaryoitic Cells: Effect of Nuclear Signal Sequence on Protein Activity and Nuclear Documentation", *Nucleic Acid Research*, Vol. 20, pp. 1785-1791;
	AR		Seipel, K., et al., (1992) "Different activation domains stimulate transcription from remote ('enhancer') and proximal ('promoter') positions", *The EMBO Journal*, Vol. 11, No. 13, pp. 4961-4968;
	AS		Epstein-Baak, R., et al., (1992) "Inducible Transformation of Cells from Transgenic Mice Expressing SV40 under *Lac* Operon Control", *Cell Growth & Differentiation*, Vol. 3, pp. 127-134;
	AT		Gossen, M., and Bujard, H., (1992) "Tight control of gene expression in mammalian cells by tetracycline-responsive promoters", *Proceedings of the National Academy of Science*, Vol. 89, pp. 5547-5551;
	AU		Bradley, A., (1991) "Modifying the mammalian genome by gene targeting", *Current Opinion in Biotechnology*, Vol. 2, pp. 832-829;
	AV		Wyborski, D.L., and Short, J.M., (1991) "Analysis of Inducers of the *E. Coli* Lac Repressor System in Mammalian Cells and Whole Animals", *Nucleic Acid Research*, Vol. 19, pp. 4647-4653;

Examiner	Date Considered

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Attachment A

APPLICANT FACSIMILE OF FORM PTO-1449 REV 7-80	U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTY DOCKET NO.	SERIAL NO.
		BBI-009C4CN	09/241,347
LIST OF PUBLICATIONS CITED BY APPLICANT (Use several sheets if necessary)		APPLICANT: Hermann Bujard and Manfred Gossen	
		FILING DATE: February 2, 1995	GROUP: 1632

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	BA		Degenkolb, J., et al., (1991) "Structural Requirements of Tetracycline-Tet Repressor Interaction: Determination of Equilibrium Binding Constants for Tetracycline Analogs with the Tet Repressor", *Antimicrobial Agents and Chemotherapy*, Vol. 35, No. 8, pp. 1591-1595;
	BB		Baim, S.B., et al., (1991) "A chimeric mammalian transactivator based on the *lac* repressor that is regulated by temperature and isopropyl β-D-thiogalactopyranoside", *Proceedings of the National Academy of Science*, Vol. 88, pp. 5072-5076;
	BC		Gatz, C., et al., (1991) "Regulation of a modified CaMV 35S promoter by the Tn *10*-encoder Tet receptor in transgenic tobacco", *Mol. Gen. Genet.*, Vol. 227, No. 2, pp. 229-237;
	BD		Wissmann, A., et al., (1991) "Selection for Tn*10* Tet Repressor Binding to *tet* Operator in *Escherichia coli*: Isolation of Temperature-Sensitive Mutants and Combinatorial Mutagenesis in the DNA Binding Motif", *Genetics*, Vol. 128, pp. 225-232;
	BE		Labow, M.A., et al., (1990) "Conversion of the *lac* Repressor into an Allosterically Regulated Transcriptional Activator for Mammalian Cells", *Molecular and Cellular Biology*, Vol. 10, No. 7, pp. 3343-3356;
	BF		Deuschle, U., et al., (1989) "Regulated expression of foreign genes in mammalian cells under the control of coliphage T3 RNA polymerase and *lac* repressor", *Proceedings of the National Academy of Science*, Vol. 86, pp. 5400-5404;
	BG		Capecchi, M.R., (1989) "Altering the Genome by Homologous Recombination", *Science*, Vol. 244, pp. 1288-1292;
	BH		Mermod, N., et al., (1989) "The Proline-Rich Transcriptional Activator of CTF/NF-I Is Distinct from the Replication and DNA Binding Domain", *Cell*, Vol. 58, 741-753;
	BI		Mansour, S.L., et al., (1988) "Disruption of the proto-oncogene *int*-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes", *Nature*, Vol. 336, pp. 348-352;
	BJ		Gatz, C., and Quail, P.H., (1988) "Tn*10*-encoded *tet* repressor can regulate an operator-containing plant promoter", *Proceedings of the National Academy of Science*, Vol. 85, pp. 1394-1397;
	BK		Figge, J., et al., (1988) "Stringent Regulation of Stably Integrated Chloramphenicol Acetyl Transferase Genes by *E. coli lac* Repressor in Monkey Cells", *Cell*, Vol. 52, 713-722;
	BL		Triezenberg, S.J., et al., (1988) "Functional dissection of VP16, the *trans*-activator of herpes simplex virus immediate early gene expression", *Genes & Development*, Vol. 2, pp. 718-729;
	BM		Courey, A.J., and Tjian, R., (1988) "Analysis of Sp1 *In Vivo* Reveals Multiple Transcriptional Domains, Including a Novel Glutamine-Rich Activation Motif", *Cell*, Vol. 55, pp. 887-898;
	BN		Tovar, K., et al., (1988) "Identification and nucleotide sequence of the class E *tet* regulatory elements and operator and inducer binding of the encoded purified Tet repressor", *Mol. Gen. Genet.*, Vol. 215, pp. 76-80;
	BO		Altschmied, L. et al., (1988) "A threonine to alanine exchange at position 40 of Tet repressor alters the recognition of the sixth base pair of *tet* operator from GC to AT", *The EMBO Journal*, Vol. 7, No. 12, pp. 4011-4017;
	BP		Brown, M., et al., (1987) "*lac* Repressor Can Regulate Expression from a Hybrid SV40 Early Promoter Containing a *lac* Operator in Animal Cells", *Cell*, Vol. 49, pp. 603-612;
	BQ		Hu,M.C-T and Davidson, N., (1987) "The Inducible *lac* Operator-Repressor System Is Functional in Mammalian Cells", *Cell*, Vol. 46, pp. 555-566;
	BR		Smithies, O., et al., (1985) "Insertion of DNA sequences into the human chromosomal β-globin locus by homologous recombination", *Nature*, Vol. 317, pp. 230-234;
	BS		Boshart, M., et al., (1985) "A Very Strong Enhancer Is Located Upstream of an Immediate Early Gene of Human Cytomegalovirus", *Cell*, Vol. 41, No. 2, pp. 521-530;
	BT		Postle, K., et al., (1984) "Nucleotide sequence of the repressor gene of the TN*10* tetracycline resistance determinant", *Nucleic Acid Research*, Vol. 12, No. 12, pp. 4849-4863;
	BU		Unger, B., et al., (1984) "Nucleotide sequence of the gene, protein purification and characterization of the pSC101-encoded tetracycline resistance-gene-repressor", *Gene*, Vol. 31, pp. 103-108;

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LIST OF PUBLICATIONS CITED BY APPLICANT (Use several sheets if necessary)		BBI-009C4CN	09/241,347
		APPLICANT: Hermann Bujard and Manfred Gossen	
		FILING DATE: February 2, 1995	GROUP: 1638

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	CA		Unger, B., et al., (1984) "Nucleotide sequence of the repressor gene of the RA1 tetracycline resistance determinant: structural and functional comparison with three related Tet repressor genes", *Nucleic Acid Research*, Vol. 12, No. 20, pp. 7693-7703;
	CB		Waters, S.H, et al., (1983) "The tetracycline resistance determinants of RP1 and Tn*1721*: nucleotide sequence analysis", *Nucleic Acid Research*, Vol. 11, No. 17, pp. 6089-6105;
	CC		Hillen, W., and Schollmeier, K., (1983) "Nucleotide sequence of the Tn*10* encoded tetracycline resistance gene", *Nucleic Acid Research*, Vol. 11, No. 2, pp. 525-539;
	CD		Brent, R. and M. Ptashne (1984) "A Bacterial Repressor Protein or a Yeast Transcriptional Terinator Can Block Upstream Activation of A Yeast Gene" *Nature* 312:612-615;
	CE		Brent R. and M. Ptashne (1985) "A Eukaryotic Transcriptional Activator Bearing the DNA Specificity of a Prokaryotic Repressor" *Cell* 43:729-736;
	CF		Baniahmad, A. et al. (1992) "A Transferable Silencing Domain Is Present In the Thyroid Hormone Receptor, In the v-erbA Oncogene Product and In the Retinoic Acid Receptor" *The EMBO Journal* 11(3):1015-1023;
	CG		Sauer, F. and H. Jäckle (1993) "Dimerization and the Control of Transcription by Krüppel" *Nature* 364:454-457;
	CH		Licht, J. et al. (1990) "Drosophila Krüppel Protein is a Transcriptional Repressor" *Nature* 346:76-79;
	CI		Herschbach B. and A. Johnson (1993) "Transcriptional Repression In Eukaryotes" *Annu. Rev. Cell Biol.* 9:479-509;
	CJ		Renkawitz R. (1990) "Transcriptional Repression In Eukaryotes" *TIG* 6(6):192-193;
	CK		Resnitzky D. (1994) "Acceleration of the G1/S Phase Transition by Expression of Cyclins D1 and E with an Inducible System" *Molecular and Cellular Biology* 14(3):1669-1679;
	CL		Furth P. (1994) "Temporal Control of Gene Expression in Transgenic Mice By A Tetracycline-Responsive Promoter" *Proc. Natl. Acad. Sci. USA* 91:9302-9306;
	CM		Wimmel A. et al. (1994) "Inducible Acceleration of G1 Progression Through Tetracycline-Regulated Expression of Human Cyclin E" *Oncogene* 9:995-997
	CN		Ackland-Berglund, C.E. and Leib, D.A.. (1995) "Efficacy of Tetracycline-Controlled Gene Expression Is Influenced by Cell Type" *BioTechniques* 18(2):196-200;
	CO		Gossen M. and B. Hermann (1993) "Anhydrotetracycline, A Novel Effector of Tetracycline Controlled Gene Expression Systems In Eukaryotic Cells" *Nucleic Acids Research* 21(18):4411-4412;
	CP		Buckbinder L. et al. (1994) "Gene Regulation by Temperature-Sensitive p53 Mutants: Identification of p53 response genes" *Proc. Natl. Acad. Sci. USA* 91:10640-10644;
	CQ		Yarranton G. (1992) "Inducible Vectors For Expression In Mamalian Cells" *Current Opinion in Biotechnology* 3:506-511;
	CR		Gossen et al. (1994) "Inducible Gene Expression Systems For Higher Eukaryotic Cells" *Current Opinion in Biotechnology* 5:516-520;
	CS		Weinmann P. et al. (1994) "A Chimeric Transactivator Allows Tetracycline-Responsive Gene Expression in Whole Plants" *The Plant Journal* 5(4):559-569;
	CT		Pescini R. et al. (1994) "Inducible Inhibition of Eukaryotic Gene Expression" *Biochemical and Biophysical Research Communications* 202(3):1664-1667;
	CU		Fishman G. et al. (1994) "Tetracycline-Regulated Cardiac Gene Expression in Vivo" *J. Clin. Invest.* 93:1864-1868;

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FOREIGN PATENT DOCUMENTS

		DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES	TRANSLATION NO
	AB	WO 94/29442	12/94	PCT				

OTHERS (including Author, Title, Date, Pertinent Pages, Etc.)

	AC		Cowell, "Repression versus activation in the control of gene transcription," *Trends in Biochemical Sciences*, 19:1, 38-42 (1994);
	AD		Deuschle et al., "Tetracycline-reversible silencing of eukaryotic promoters," *Mol. Cell. Biol.*, 15:4, 1907-1914 (1995);
	AE		Gatz et al., "Stringent repression and homogeneous de-repression by tetracycline of a modified CaMV 35S promoter in intact transgenic tobacco plants," *The Plant Journal*, 2:3, 397-404 (1992);
	AF		Gossen et al., "Exploiting prokaryotic elements for the control of gene activity in higher eukaryotics," Keystone Symposium on Gene Therapy and Molecular Medicine, Steamboat Springs, Colorado, *Journal of Cellular Biochemistry*, Supplement 0 (21A), Abstract no. C6-220, 355 (1995);
	AG		Gossen et al., "Transcriptional activation by tetracyclines in mammalian cells," *Science*, 268:5218, 1766-1769 (1995);
	AH		Liang et al., "Enhanced and switchable expression systems for gene-transfer," Keystone Symposium on Gene Therapy and Molecular Medicine, Steamboat Springs, Colorado, *Journal of Cellular Biochemistry*, Supplement 0 (21A), Abstract no. C6-220, 379 (1995).
	AI		Agarwal, M.L. et al., "p53 Controls Both the G_2/M and the G_1 Cell Cycle Checkpoints and Mediates Reversible Growth Arrest in Human fibroblasts," *Proc. Natl. Acad. Sci. USA*, 92: pp. 8493-8497 (1995);
	AJ		Bergman, M. et al. "Overexpressed Csk Tyrosine Kinase Is Localized in Focal Adhesions, Causes Reorganization of $\alpha_V\beta_5$ Integrin, and Interferes with HeLa Cell Spreading", *Molecular and Cellular Biology*, 15, No. 2, pp. 711-722 (1995);
	AK		Cayrol, C. et al. "Identification of Cellular Target Genes of the Epstein-Barr Virus Transactivator Zta: Activation of Transforming Growth Factor βigh3 (TGF-βigh3) and TGF-β1", *Journal of Virology*, 69, No. 7, pp. 4206-4212, (1995);
	AL		Chen, Y.Q. et al. "Tumor Suppression by *p21*^{WAF11}", *Cancer Research*, 55, pp. 4536-4539, (1995);
	AM		Dhawan, J. et al. "Tetracycline-Regulated Gene Expression Following Direct Gene Transfer into Mouse Skeletal Muscle", *Somatic Cell and Molecular Genetics*, 21, No. 4, pp. 233-240, (1995);

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		DOCUMENT NUMBER	DATE	COUNTRY	CLASS	SUBCLASS	TRANSLATION YES	NO

OTHERS (including Author, Title, Date, Pertinent Pages, Etc.)

	AN		Efrat, S. et al. "Conditional Transformation of a Pancreatic β-Cell Line Derived From Transgenic Mice Expressing A Tetracycline-Regulated Oncogene" *Proc. Natl. Acad. Sci. USA*, 92, pp. 3576-3580 (1995);
	AO		Gjetting, T. et al. "Regulated Expression of the Retinoblastoma Susceptibility Gene in Mammary Carcinoma Cells Restores Cyclin D1 Expression and G_1-Phase Control", *Biol. Chem. Hoppe-Seyler*, 376, pp. 441-446 (1995);
	AP		Haase, S.B. et al. "Transcription Inhibits the Replication of Autonomously Replicating Plasmids in Human Cells", *Molecular and Cellular Biology*, 14, No. 4, pp. 2516-2524 (1994);
	AQ		Hennighausen, L. et al. "Conditional Gene Expression in Secretory Tissues and Skin of Transgenic Mice Using the MMTV-LTR and the Tetracycline Responsive System", *Journal of Cellular Biochemistry*, 59, pp. 463-472, (1995);
	AR		Howe, J.R. et al. "The Responsiveness of a Tetracycline-Sensitive Expression System Differs in Different Cell Lines", *The Journal of Biological Chemistry*, 270, No. 23, pp. 14168-14174, (1995);
	AS		Miller, K. et al. "The Function of Inducible Promoter Systems in F9 Embryonal Carcinoma Cells", *Experimental Cell Research*, 218, pp. 144-150, (1995);
	AT		Passman, R.S. et al., "Regulated Expression of Foreign Genes In Vivo After Germline Transfer", *J. Clin. Invest.*, 94, pp. 2421-2425 (1994)
	AU		Sopher, B.L. et al., "Cytotoxicity Mediated By Conditional Expression of a Carboxyl-Terminal Derivative of the β-Amyloid Precursor Protein", *Molecular Brain Research*, 26, pp. 207-217, (1994);
	AV		Wu, Z. et al. "Conditional Ectopic Expression of C/EBPβ in NIH-3T3 Cells Induces PPARγ and Stimulates Adipogenesis", *Genes & Development*, 9, pp. 2350-2363, (1995).

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